Universität Ulm

Neurogenesis in the brain of the Marbled crayfish

Dissertation

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Silvia Sintoni

aus Forlì in Italien

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Amtierender Dekan: Prof. Dr. Axel Groß

Erster Gutachter: Prof. Dr. Harald Wolf

Zweiter Gutachter: Prof. Dr. Steffen Harzsch

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A te Sarah,
che sei la vita stessa.

A te Davide,
che hai reso tutto questo possibile.

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Chapter 1

General Introduction

1.1 INTRODUCTION

1.1.1. History of Crustacean Neuroanatomy

The first reference to the crayfish in scientific literature is in Aristotle's "History of Animals" (350 B.C. Translated by D'Árcy Wentworth Thompson) where small Astaci were reported to "breed in the rivers". The term Astaci included both the crayfish and the lobster. In more recent times, the French naturalist Henry Milne Edwards (1800-1885) dedicated a book to the biology of Crustacea (Histoire naturelle des Crustaces, 3 volumes, 1837-1841), considered one of the most important and fundamental carcinological publication of the first half of the 19th century.

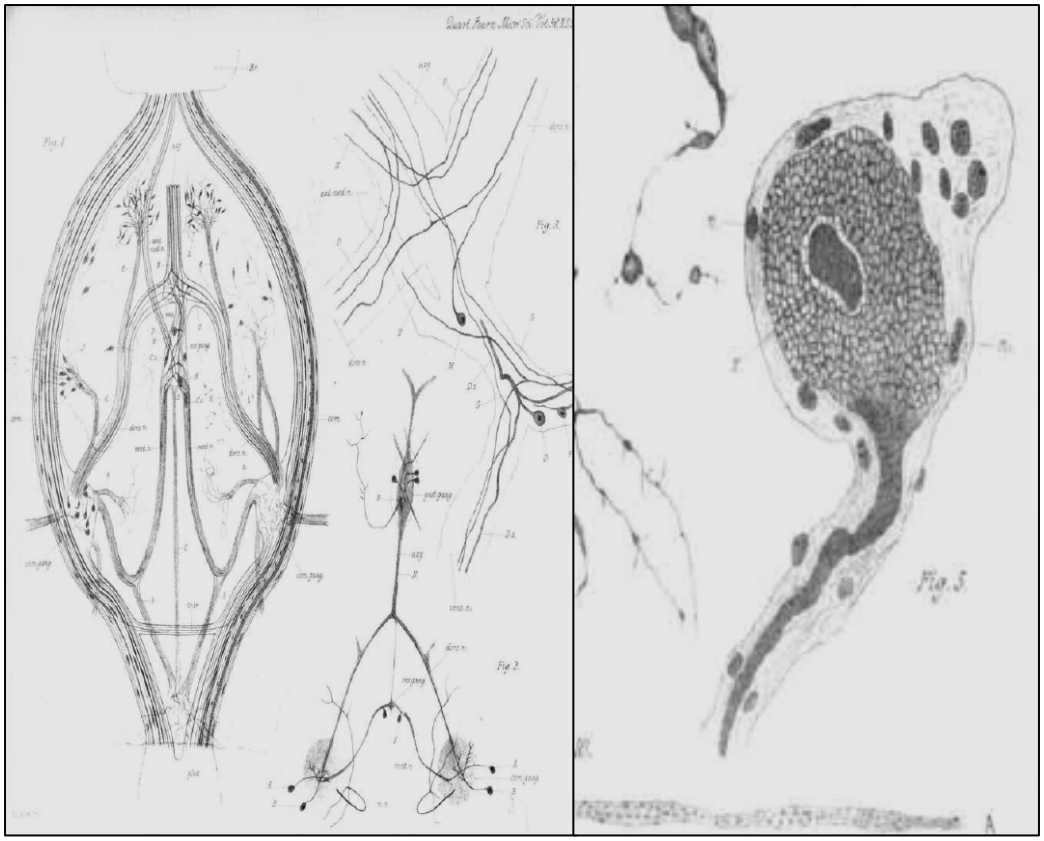


Fig. 1.1- Sketches showing the main features of the nervous system of an adult lobster (*Astacus fluviatilis*). On the left: aesophageal commisures. On the right: ganglion cell showing connective tissue sheath with its nuclei (from Allen, 1896).

Edgar Johnson Allen (1866-1942), a british polychaetologist and phytoplankton researcher, director of the Plymouth laboratory, published results on the brain of lobster

embryos (Allen 1894, 1896). His career started by collecting Crustacea in Antigua and that was his first step into science after receiving a studentship by the Royal Society. His work in the beginning was on the nervous system of the embryonic lobster and later on crab nervous system and body organization. His papers are detailed descriptions of single cells and fibers of the nervous system (Fig. 1.1, 1.2).

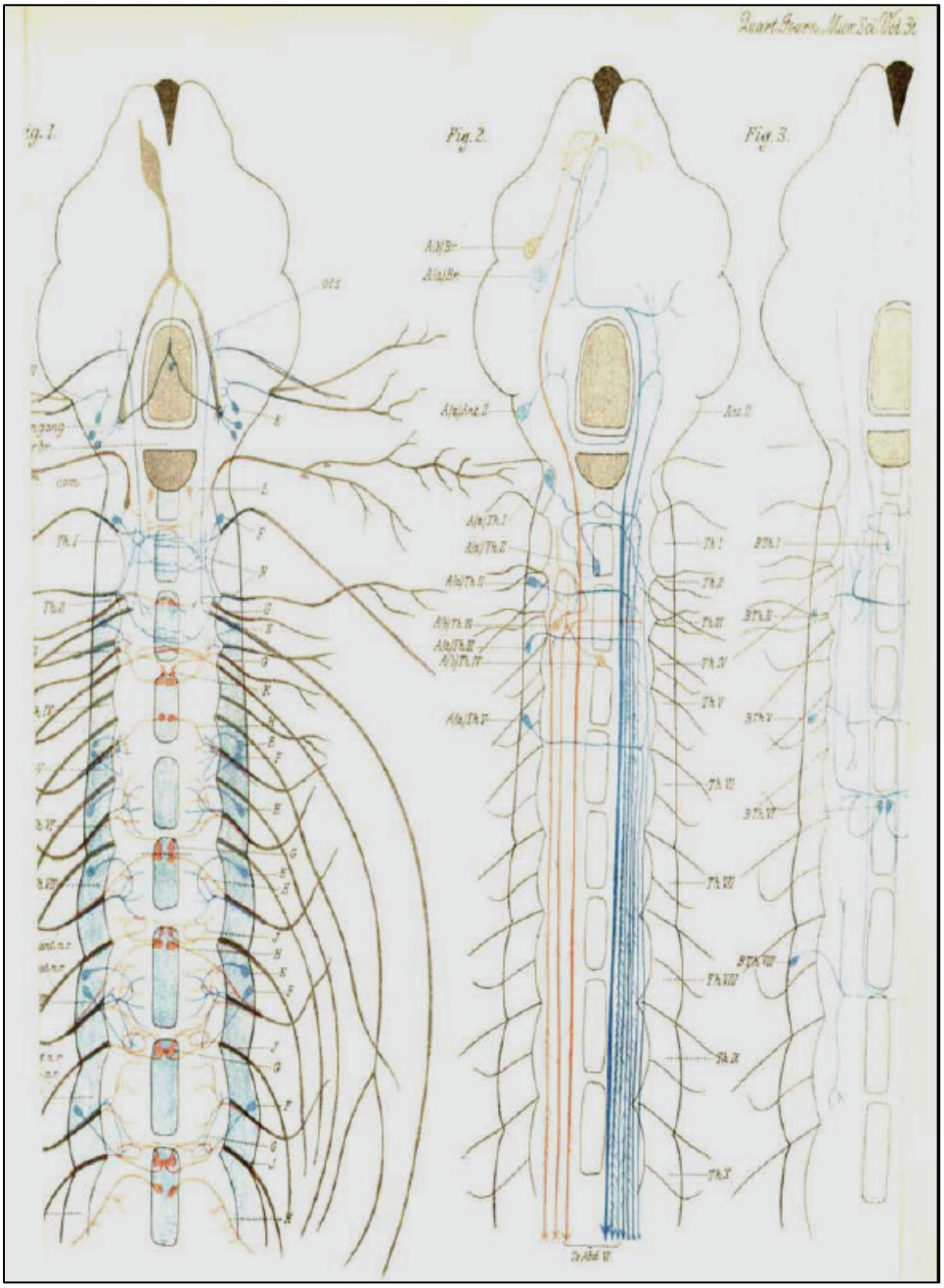


Fig. 1.2- Sketch showing the main features of the nervous system of an adult lobster (*Astacus fluviatilis*). View of the stomatograstric system with the central nervous system (from Allen, 1896).

In the beginning of 20th century, first comparative studies appeared. Helm, Holmgren and his student Hanström published various works on invertebrates' brain, in particular by emphasizing comparisons among different groups (Hanström, 1925, 1947) (Fig. 1.3) (Hanström, 1926. In: Strausfeld, 2009).

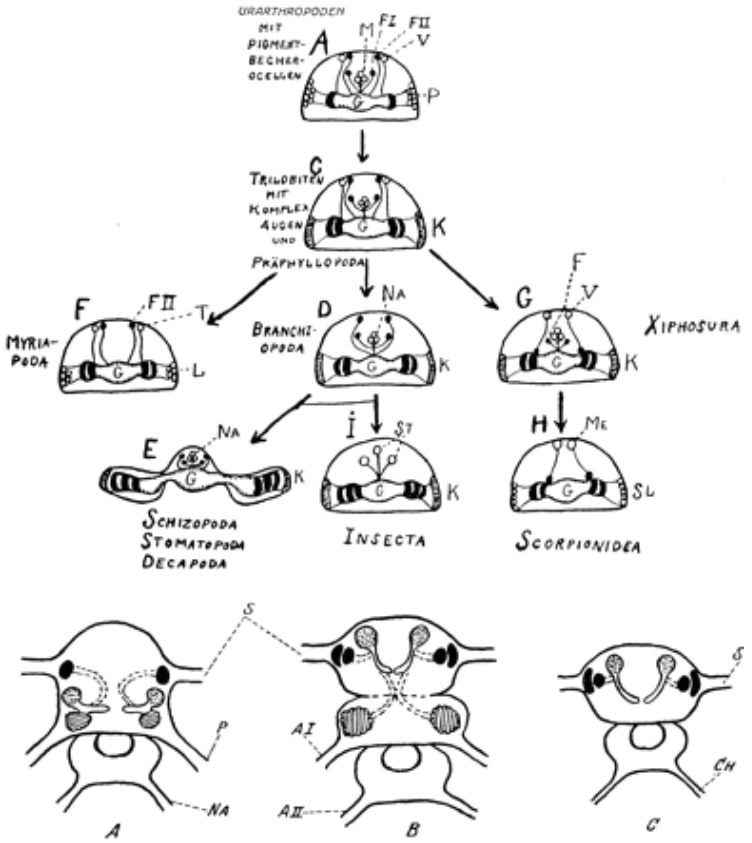
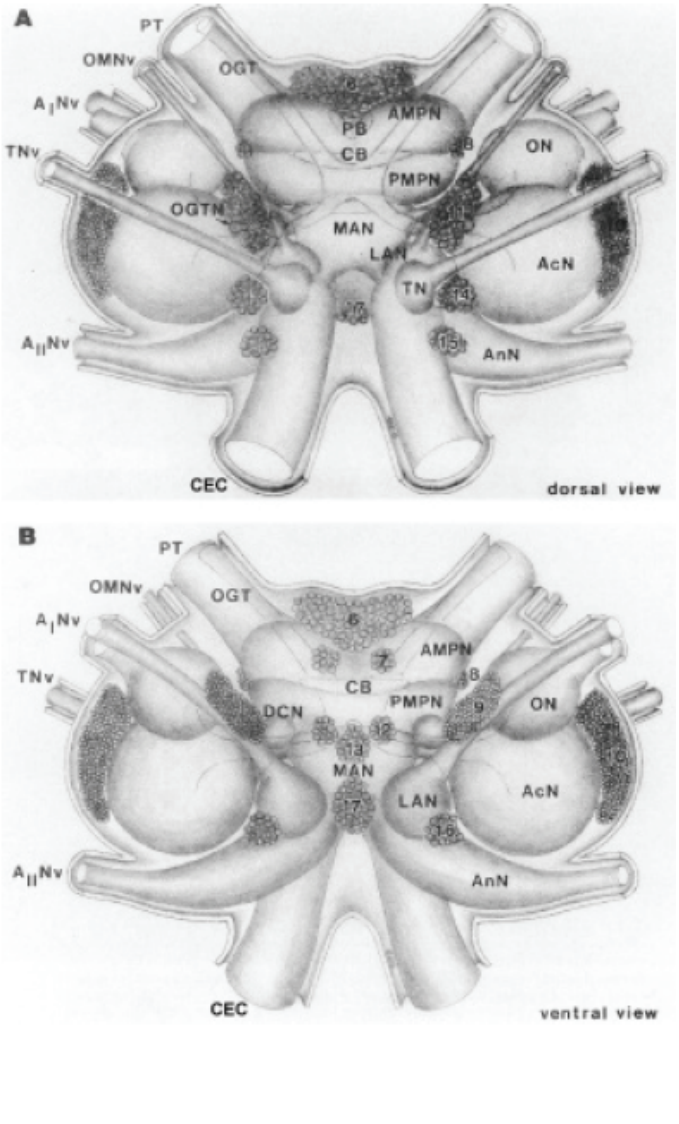


Fig. 1.3- Hanström's classic 1926 paper claiming arthropod monophyly on the basis of observed similarities among visual systems. In this phylogram, based on brain morphology, both the Crustacea and Tracheata are paraphyletic. In the bottom: A: annelids; B: arthropods C: araneans.

In particular, the Swedish neuroanatomist Nils Holmgren (1916) (in: Strausfeld, 2009) was the first one, together with his pupil, that, instead of external morphological criteria, relied on neural characters to infer evolutionary relationships across arthropods. Both these two swedish researchers set the base for the field of research that combines neuroanatomy and phylogeny and that will be successively called "Neurophylogeny" (Harzsch 2002a, 2006). Unfortunately, due to the absence of a standard nomenclature, misunderstandings about anatomical structures were reported afterwards (discussed by Sandeman et al., 1992).



Nerves
AMNv anterior median nerve
OMNv oculomotor nerve
A_INv antenna I nerve
A_{II}Nv antenna II nerve
StNv statocyst nerve
TNv tegumentary nerve
PMnv posterior median nerve
RNv recurrent nerve
MN median nerve

Brain divisions and neuropils

Optic ganglia
L Lamina (lamina ganglionaris)
M Medulla (medulla externa)

Lateral protocerebrum
Lo Lobula (medulla interna)
MT Medulla terminalis (medulla terminalis) which is composed of the
DMN diamedullary neuropil, of the
HN hemiellipsoid body and of eight glomeruli centrales.

Median Protocerebrum
AMPN anterior medial protocerebral neuropil
PMPN posterior medial protocerebral neuropil
CC Central complex which is composed of the
PB protocerebral bridge and the
CB central body and the
LL lateral lobes

Deutocerebrum
ON olfactory lobe/neuropil
LAN lateral antenna I neuropil
MAN median antenna I neuropil
AcN acessory lobe/neuropil
DCN deutocerebral commissure neuropil
OGTN olfactory globular tract neuropil

Tritocerebrum
AnN antenna II neuropil
TN tegumentary neuropil

Tracts and commissures
OT optic tract
PT protocerebral tract
OGT olfactory globular tract
DC deutocerebral commissure
CEC circumesopgageal connectives
PEC postesophageal commissure
LC labral commissure

Other abbreviations
A1 antenna I
A2 antenna II
CE compound eyes
EG esophageal ganglion
LB labrum
SL statolith

Fig. 1.4- Graphic reconstruction and nomenclature of the brain of a crayfish proposed by Sandeman and colleagues (1992) and Strausfeld and Nässel (1981, optic ganglia).

Tsvileneva and Titova (1985) described the brain structure of decapod crustaceans by recalling the nomenclature used by Helm, in the attempt to overcome such mistakes in nomenclature. Finally, in 1992, Sandeman and colleagues proposed a common nomenclature for homologous nerve roots, brain regions, tracts, commisures, neuropils and cell body cluster by illustrating the cerebral ganglia (brains) of crayfish (Fig. 1.4), crabs and spiny lobsters. Further contributions to our knowledge of the brain structure were provided e.g. by Strausfeld and Nässel (1981), Sandeman (1982), Nässel and

Elofsson (1987), Blaustein et al. (1988), Beltz (1990), Sandeman et al. (1993), Sandeman and Scholtz (1995). The following general description of the crustacean nervous system is based on these works. The nervous system is made of a brain and a ventral nerve cord (Fig. 1.5).

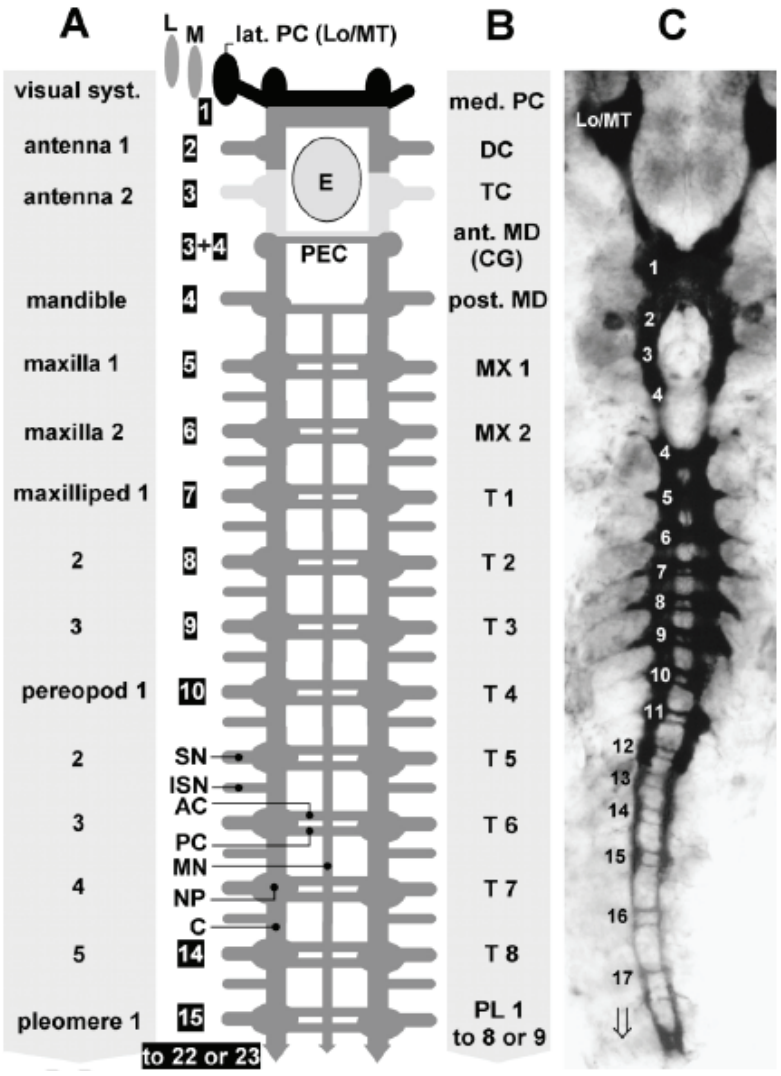


Fig. 1.5- A, B: Schematic representation of the segmental composition of the malacostracan central nervous system. The peripheral structures associated with the segments are listed in A, the names of the neuromeres in B. C: Synapsin-immunoreactivity in an embryo of the shrimp *Palaemonetes argentinus*. Note that not all pleon ganglia are formed at this stage. Abbr.: AC: anterior commissure, C: lateral connective, CG: 'commissural ganglion', DC: deutocerebrum, E: esophagus, ISN: intersegmental nerve, L: lamina, Lo: lobula, ant./post. MD: anterior and posterior parts of the mandibular neuromere, MN: median nerve or connective, MT: medulla terminalis, MX1, 2: neuromeres of maxilla one and two, NP: neuropil, PC: posterior commissure, lat./med. PC: lateral and medial parts of the protocerebrum, PEC: postesophageal commissure, PL 1–9: pleon neuromeres one to nine, SN: segmental nerve, T 1–8: thoracic neuromeres one to eight, TC: tritocerebrum (from Harzsch, 2003a).

The brain is a complex assemblage of cell body clusters and various neuropil compartments arranged in distinct spatial relationships (Strausfeld 1976, Harzsch 2006). The term neuropil usually refers to fibrous regions of the brain composed of tangles of nerve processes and non neuronal elements such as glia cells and blood vessel (Maynard, 1962). The brain is composed of three main divisions: protocerebrum, deutocerebrum and tritocerebrum representing the three segmental units, neuromeres, that are fused to form

the brain of decapods (Fig. 1.4, 1.5). The two optic neuropils are located within the eye stalks and belong to the protocerebrum as well as a unpaired neuropil, the central body, innervated by interneurons, some of them serotonergic. The lateral eyes are made of several units and are associated to three optic ganglia, the medulla, lamina and lobula that provide inputs into the protocerebrum. The deutocerebrum and its connection to the olfaction will be extensively described in Chapter 5. The tritocerebrum is the third and most caudal of the segmental brain units. The appendages on the third segment are the second antennae that carry mechanoreceptive sensilla capable of detecting very small disturbances in the surrounding water. The tritocerebrum is caudally connected with the neuromere associated with the mandible which is followed the neuromeres of the maxilla one and two. The ventral nerve cord runs along the body on the ventral side and is made of a series of ganglia, one in each segment of the body. Each ganglion is a bilaterally symmetrical structure, the two hemiganglia are linked across the midline by transverse commissures. Neighbouring ganglia are linked by paired longitudinal connectives (Fig. 1.5).

1.1.1.1. Embryonic development of the brain

Strictly speaking about Malacostraca (e.g. crabs, crayfish and lobster), the anatomy of the embryonic central nervous system (CNS) and its development have been investigated by using different approaches ranging from classical histology (Elofsson 1969, Scholtz 1992, Harzsch and Dawirs 1993, Helluy et al. 1995, 1996, Rotllant et al. 1994, 1995, Gerberding 1997) to more detailed techniques like the use of neuron-specific antibodies (Meier and Reichert 1990, Garzino and Reichert 1994), phalloidin-staining (Whitington et al. 1993, Scholtz 1995a and b, Sandeman and Sandeman 2003, Vilpoux et al. 2006), intracellular tracing techniques (Whitington et al. 1993, Gerberding and Scholtz 1999, 2001), immunolocalization of synapsin (Harzsch et al. 1997), Engrailed (Scholtz 1995a, b, Sintoni et al. 2007), FMFR-amide related peptides (Harzsch and Dawirs, 1996b), GABA and glutamate (Foa and Cooke, 1998) and serotonin (Beltz et al., 1992). The external morphology and development of decapod embryos (including the main features of the body and head formation) were analyzed and staged by Sandeman and Sandeman (1991; *Cherax destructor*), Seitz et al (2005; Marbled crayfish) and Alwes and Scholtz

(2006; Marbled crayfish). The detailed mode by which an embryo and, in particular, its brain develop during embryogenesis will be treated separately in the next chapter on neurogenesis (Chapter 2).

1.1.1.2. The dispute on arthropod phylogeny

The position of Crustacea within Arthropoda is a matter of intense and continual debate. The phylum of arthropods includes four main groups: Insecta, Crustacea (divided into Malacostraca and Entomostraca), Myriapoda and Chelicerata and relationships among them are still an unresolved question. The issue has been challenged by different approaches such as anatomical comparisons, molecular analysis and paleontology. For many years, the so called "Tracheata-Atelocerata" hypothesis that supported the myriapod-insect clade was the most popular view (Kraus 1997, 2001, Walossek and Müller 1998, Walossek 2003, Klass and Christensen 2001). Afterwards, due to molecular and developmental studies, the Tracheata concept was discarded by many phylogenists in favor of the Tetraconata hypothesis (Dohle 2001, Friedrich and Tautz 1995, Boore et al. 1998, Richter 2002) that suggests a sister group relationship between crustaceans and insects. The "neural phylogeny" (Paul, 1989) or, better, "neurophylogeny" (Harzsch, 2006) attempts to contribute to the controversy by using shared neuronal characters, in order to infer evolutionary relationships. Actually, this discipline is not new. First observations on differences of brain anatomy were observed in 1878 by the German naturalist Flögel "who 130 years ago pioneered comparative arthropod neuroanatomy" by identifying comparable elements in different taxa studies of the insect supraoesophageal ganglia (In: Strausfeld and Seyfarth, 2008). "Flögel recognized the interpretative power resulting from observations across various species", and he also provided a baseline for a nomenclature of insect brain structures (Strausfeld and Seyfarth, 2008). As mentioned before (see Strausfeld, 2009), in 1916, Holmgren published the first work on neurophylogeny on the Arthropoda, not based on external anatomical traits but on characters of the nervous system. Holmgren (and subsequently Hanström, 1926) declared the arthropods to be monophyletic relying on the assumption that neuropils of visual system were equivalent in all arthropods (in: Strausfeld, 2009). A similar view, based on visual system structure, was re-evaluted by Paulus (1979), in totally contrast to the

hypothesis brought up by Manton (1977. In: Turbeville et al., 1991) that arthropods are polyphyletic due to the fact that features of development, functional anatomy and morphology of living arthropods are so distinct that arthropods must lack a common ancestor. Three independent groups ([Insecta + Myriapoda] + Crustacea + Chelicerata) were in fact supposed to represent three monophyletic groups, utterly unrelated to each other. Now the view has changed and the common opinion is again a monophyly of Arthropoda (Turbeville et al. 1991, Panganiban et al. 1995, Giribet et al. 1996, 2001, 2005, Friedrick and Tautz 2001) but the origin of insects and relationships among the other groups are still open to debate. According to Bushbeck (2000), neural characters generally maintain phylogenetic information well. This author discussed reliability of neuronal characters of the fly visual system, in particular uniquely identifiable neurons, which are cells or cell types that can be confidently recognized as homologues among different species and thus compared. Parsimony analysis resulted in a phylogenetic hypothesis that favors previously suggested phylogenetic relationships.

In the past years, more and more data have accumulated on the arthropod nervous system in order to expand the knowledge in particular into the phylogenetic relationships of Crustacea (reviews by Whitington 1996, Nilsson and Osorio 1997, Whitington and Bacon 1997, Strausfeld 1998, Paulus 2000, Dohle 2001, Richter 2002, Harzsch 2006). What has been investigated so far from an evolutionary point of view are: the visual system (Melzer et al 1997, 2000, Richter 1999, Paulus 2000, Hafner and Tokarski 2001, Harzsch et al 1999a, Harzsch and Walossek 2001, Harzsch 2002b, Wildt and Harzsch 2002, Harzsch and Hafner 2006), the morphology of the brain (Strausfeld 1998, Utting et al 2000, Harzsch and Glötzner 2002, Loesel et al 2002), the ventral nerve cord (Thomas et al. 1984, Whitington 1996, Whitington and Bacon 1997, Gerberding and Scholtz 1999, 2001, Fabritius-Vilpoux et al. 2008), neuroblasts proliferation (Harzsch et al 1998, Harzsch 2001, reviewed by Harzsch 2002a). Furthermore, the morphology of individually identified neurons in the ventral nerve cord has been compared (Wiens and Wolf 1993, Kutsch and Breidbach 1994, Kutsch and Heckmann 1995, Wolf and Harzsch 2002a, b, Dircksen 1998, Ungerer and Scholtz 2008), as well as *engrailed*- and *even-skipped*-expressing cells (Duman-Scheel and Patel 1999, Sintoni et al. 2007, Fabritius-Vilpoux et al. 2008), serotonin- (Harzsch and Waloszek, 2000), histamine- (Rieger and

Harzsch, 2007) and pigment-dispersing hormone- immunoreactive neurons (Harzsch et al, 2009). The techniques used in the aforementioned studies range mostly from histological sectioning to confocal laser scanning microscopy (CLSM), immunocytochemical staining and electron microscopy. Molecular dataset are available as well: phylogenetics has managed to successfully collaborate with morphology in matters of phylogeny. Various studies based on molecular techniques confirmed, for instance, the crustacean-hexapod clade: Boore et al. 1998, Shultz and Regier 2000, Friedrich and Tautz 2001, Giribet et al. 2001, 2005, Regier et al. 2005.

Neurophylogeny results coming from diverse disciplines and obtained by using different techniques converge to similar conclusions in favor of the Tetraconata hypothesis. Despite the large amount of data accumulated in the past years there is still much to do in order to get closer insight to the functional significance of brain architecture, development of the brain and relationships among different groups of arthropods. More over, further comparisons with "new" and not model organisms are tremendously required and one of the first steps towards such goal may be getting to know a still unknown crustacean species with outstanding features: for instance the Marbled crayfish.

1.1.2. The Marbled crayfish

In 1995, an unidentified decapod crustacean, of marbled appearance and unknown origin, was introduced into the German aquarium trade (Fig. 1.6) (Scholtz et al., 2003). By comparing partial sequences of two mitochondrial genes with those of other related species, Scholtz and colleagues (2003) suggested a close affinity to the species *Procambarus fallax* (living in South Florida. Dorn and Trexler, 2007), indicating an affiliation to the genus *Procambarus*. More recent findings suggested a closer similarity to *Procambarus alleni* (Souty-Grosset et al., 2006. In: Marzano, 2009), leaving the debate on identity of the Marbled crayfish still open. What was surprising about the mysterious decapod crayfish was the exclusive female morphology and thus the capability of unisexual reproduction. Usually freshwater decapods are bisexual but the Marmorkrebs (German name by Werner, 1998. In: Vogt, 2008a) show gonads, ovaries with oviducts but no ovotestis or male gonoducts, and they are able to repeatedly lay eggs all year round with intervals of ten weeks between spawning (Seitz et al, 2005).



Fig. 1.6- The Marbled crayfish (image from Seitz et al., 2005)

Vogt and colleagues (2004) could demonstrate that the Marbled crayfish is the only parthenogenetic example of about 10,000 decapod species known to date. Parthenogenesis is, in fact, not unusual among crustacean such as Cladocera and Ostracoda but untypical in Decapoda (Seitz et al., 2005). Crustaceans and insects can be infected by the bacterium *Wolbachia* that induces feminization and parthenogenesis but, by using molecular probes designed for the bacterium, it was demonstrated that the Marbled crayfish is not infected and thus its parthenogenesis is genetically based (Vogt et al., 2008). Martin and colleagues (2007) (and independently Vogt et al., 2008) were able to better define the type of reproduction of the Marmorkrebs: apomixes thelyotheks. By testing 19 individuals of different generations and by using six different microsatellite markers, they discovered that all individuals showed identical results in six *loci*. No recombination occurred during oogenesis and specimens were genetically uniform. These results proved the absence of meioses and concluded that the Marmorkrebs propagates apomictically. Parthenogenesis, in general, means that growth and development of the embryo occurs without fertilization.

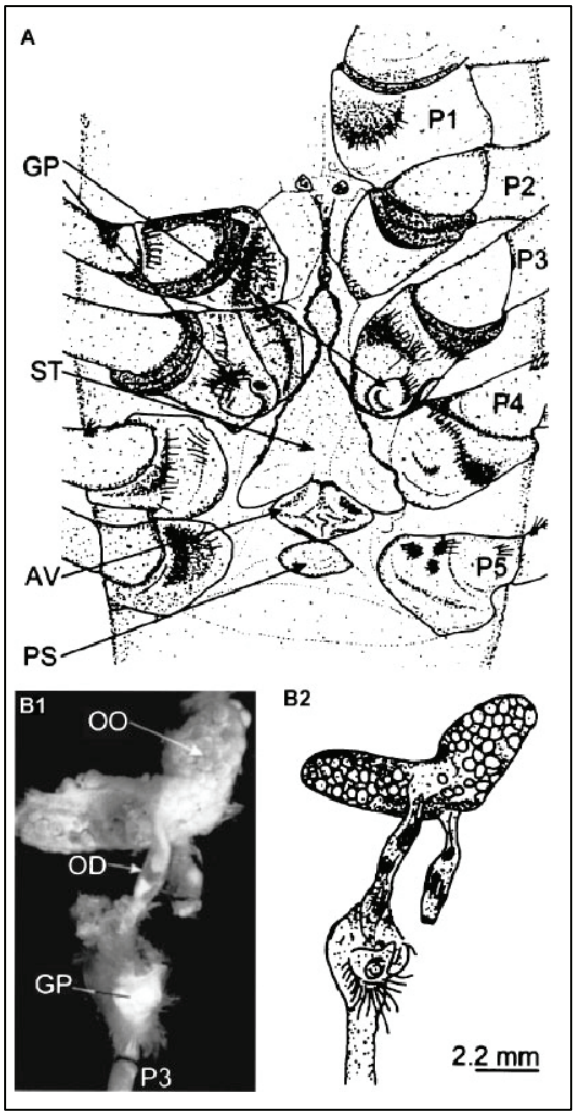


Fig. 1.7- A: ventral aspect of a female Marbled crayfish that shows female genitalia with gonopore. AV: annulus ventralis, GP: gonopores, P1-5: pereiopods one to five, PS: postannular sclerite, ST: sternum. B1, B2: ovary of a female at 56mm total length. OO: oocytes, OD: oviduct, GP: gonopore, P3: pereiopod 3. No male reproductive system is present (from Seitz et al., 2005).

When parthenogenesis give rise to males is called arrhenotoky, when it gives rise to only female is called thelytoky. Because the Marmorkrebs population is exclusively composed of females, its reproduction is clearly thelytokous (Scholtz et al., 2003). There are four main types of thelytoky, the one related to the Marmorkrebs is called apomixis. Meiosis does not occur and the offspring are genetically identical clone of the mother (Martin et al., 2007). In general, apomictic thelytoky means production of diploid daughters from unfertilized eggs. The reason why a species evolve with such mode of reproduction could be found in the increase of reproductive potential of the population due to the exclusive production of females. Moreover, cloning allows the perpetuating of the same identical genetic material generation after generation and in same cases (stable environment) this can represent a winning strategy.

1.1.2.1. Development and biology of the Marbled crayfish

The Marbled crayfish is a freshwater decapod crustacean, adapted to live at about 20-25ºC, a range of temperature which is higher compared to European species (Seitz et al., 2005). Females are mature and ready for the first reproductive event at the age of 141-255 days, a cephalotorax length of 14-21.5 mm and a weight of 0.63-2 g; larger females (25-35mm) can produce larger clutches of eggs (about 416; Seitz et al., 2005).

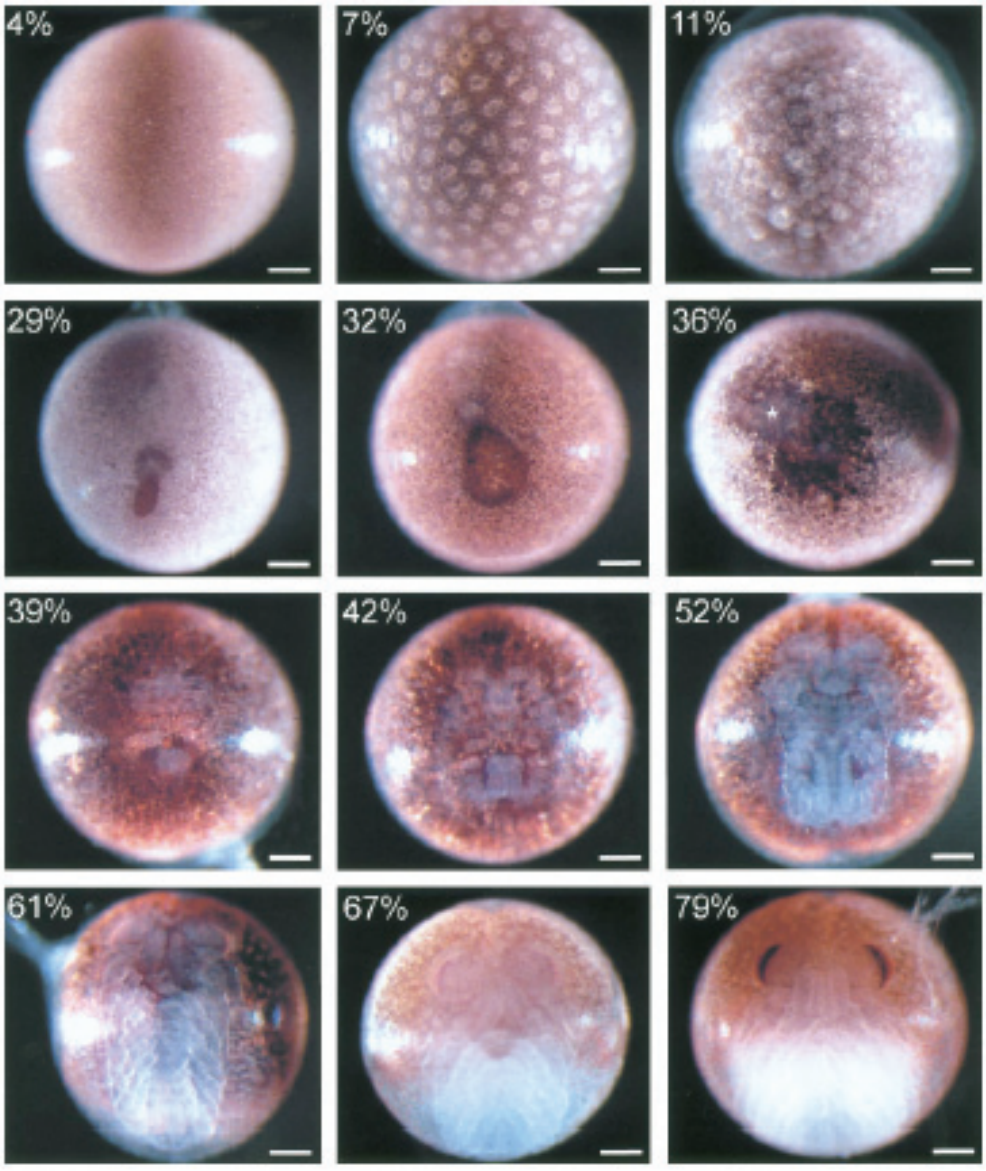


Fig. 1.8- Embryonic development of the Marbled crayfish. Percentage values represent the % of development on a normalized scale. Asterisk: embryonic tissue. Scale bars: 250µm (from Seitz et al., 2005).

Once the female deposited new eggs, the embryonic development of each embryo takes about 22 and 42 days, and it has been noticed that embryos belonging to the same clutch

of eggs do not develop in perfect synchrony (Seitz et al., 2005). The same authors established a staging system of the embryonic development, based on the percentage total time from deposition of eggs to hatchings, in order to create a valid instrument for future developmental studies (Fig. 1.8). Embryos were photographed at two-day intervals and events like gastrulation (Fig. 1.8, E29%), appearance of the germ band (Fig. 1.8, E36%), heart beat (Fig. 1.8, E42%), appearance of pigments in the carapace (Fig. 1.8, E61%) and eye pigmentation were reported (Fig. 1.8, E67%). Embryos hatch to the so called postembryonic stage (POI) (Sandeman and Sandeman 1991, Seitz et al. 2005) and have well developed eyes, full set of segments and appendage anlagen, and they are still attached to the mother's pleopods by a ligament coming from the telson (Fig. 1.9A, B).

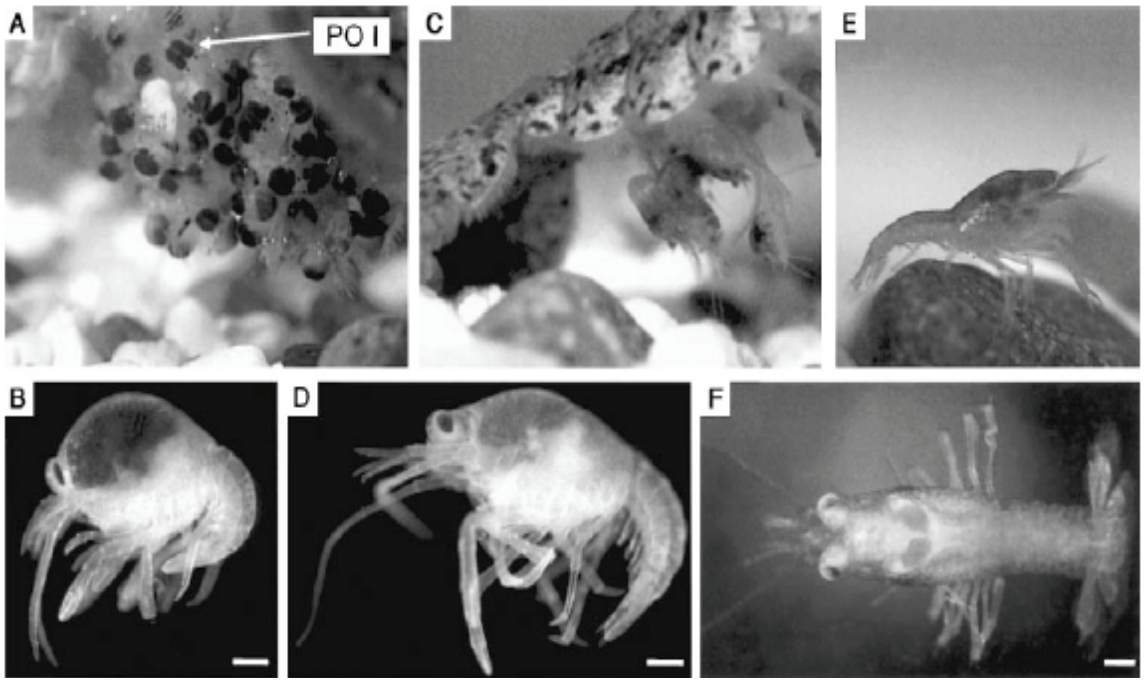


Fig. 1.9- Postembryonic development of the Marbled crayfish. A, B: newly hatched first postembryonic stages (POI). C, D: Postembryonic stages II (POII). E, F: First juvenile stage. Scale bars: 500µm (from Seitz et al., 2005).

After one to three days, the POI stage molts into the POII (postembryonic stage II) (Fig. 1.9C, D), a free animal that still clings to the mother's pleopods. Like POI, POII relies on a yolk supply stored under the dorsal carapace. After a second molt, the animals become miniatures of the adult crayfish (juvenile), able to seek food independently but still looking for protection under the mother`s pleon (Fig. 1.9E, F; Seitz et al., 2005). An interesting feature of the Marbled crayfish is that, in spite of genetic identity of the

progeny, each specimen can be individually recognized by its pigmentation pattern (Vogt et al., 2008).

1.1.2.2. Ecology of the Marbled crayfish

Due to the parthenogenetic mode of reproduction and its capability of adapt to a wide range of environmental conditions, the Marbled crayfish represents a potential ecological thread that risks to compete with native crayfish and even transmits diseases (Scholtz et al., 2003). The species in fact appears to adapt very easily at external conditions. It is sufficient to say that, in laboratory, specimens feed on almost everything and they can be raised by pellets or some vegetables. They are best cultured at 18-25ºC but they can stand temperatures even below 8ºC and above 30ºC (but not for long periods) (Seitz et al. 2005, Vogt 2008b) and, although aquatic animals, they are able to breathe also atmospheric air.
After the first records of living, wild populations in Europe (Scholtz et al., 2003), the Marbled crayfish has been found first in Madagascar (Jones et al. 2009, Kawai et al. 2009) and subsequently in Italy (Marzano et al., 2009). In 2007, in the capital city of Madagascar, an unusual crayfish was sold alive in a food market. It was then identified as *Procambarus* Marmorkrebs by molecular analysis (Jones et al., 2009). One year later, its presence was newly reconfirmed in Madagascar on the basis of morphological tracts and the presence of females only (Kawai et al., 2009). In April 2008, in Tuscany (central Italy), one single Marmorkrebs was recognized among a well adapted population of *Procambarus clarkii* (Marzano et al., 2009). Not only external characters, but also molecular distinctive traits confirmed the species. Results proved that the presence of this species is still sporadic in Italy but an adequate monitoring of its presence in the wild would be highly recommended in order to avoid additional concern on this non-indigenous species.

1.1.2.3. The Marbled crayfish as a model organism

The most popular animal models used in laboratories are the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the zebrafish *Danio rerio*, the frog *Xenopus laevis*, the chicken *Gallus gallus domesticus*, the mouse *Mus musculus* and the rat *Rattus norvegicus*. Model organisms have some characteristics in common such as

"easy culture, non-specialist living requirements and amenability for experimental manipulation, but also have one or more special features, which qualify them to be an outstanding subject of research" (Vogt, 2008a): for instance, in *C. elegans* the fate of each cell can exactly be traced and in *D. melanogaster* mutations can easily be induced to observe different phenotypes (Vogt, 2008a). Another desirable requirement is the uniformity of genetic patrimony among the offspring. Genetically identical animals are very much requested as laboratory organisms because they allow studies on development, structures, behavior and effects of environment on their biology. Moreover, genetically uniform organisms characterized by a high fertility, high reproduction rate, short generation time, breeding at any time of the year, staged developmental system, easy accessibility of all life stages for observation, manipulation or sampling, easy handling, and a complex morphology and behavior represent an even better combination of biological features. Unfortunately, such a combination of scientifically interesting aspects is not widespread in the animal kingdom but the Marmorkrebs can be considered an exception. Data presented in the previous sections showed that the development of the Marbled crayfish is more or less identical to that of other crayfish and therefore can be considered as a suitable model organism for developmental studies on malacostracan crustaceans. Decapod crustaceans are the most conspicuous aquatic invertebrates (more than 10,000 species described, 640 are freshwater crayfish) and are ecologically and economically very important (see Vogt, 2008a). In the past years, freshwater crayfish have frequently been used to investigate morphological, physiological and developmental characteristics in Crustacea. Concerning the Marbled crayfish, to my knowledge, 24 scientific articles have been published so far: Scholtz et al. 2003, Vogt and Tolley 2004, Vogt et al. 2004, 2008, Seitz et al. 2005, Alwes and Scholtz 2006, Braband et al. 2006, Vilpoux et al. 2006, Martin et al. 2007, Polanska et al. 2007, Schiewek et al. 2007, Sintoni et al. 2007, Fabritius-Vilpoux et al. 2008, Vogt 2007, 2008 a,b,c, 2009, Rieger and Harzsch 2007, Marzano et al. 2009, Farca Luna et al. 2009, Jones et al. 2009, Kawai et al. 2009, Jimenez and Faulkes 2010). A continuously updated web site by Zen Faulkes on the Marbled crayfish is available at the following website: http://marmorkrebs.org.

1.1.3. The Marbled crayfish in neurobiology and neurophylogeny: my project

As laid out before, the Marbled crayfish could reach the status of laboratory organism very soon due its outstanding biological characteristic. However, aside from the afore-mentioned studies on embryonic development and life cycle, few have dealt with aspects of neurobiology, stem cells research and neurophylogeny in this organism. In the present study, I founded all my experiments on the Marbled crayfish that resulted to be extremely suitable for these purposes. The main topic of my PhD project was the development of the brain during and after embryogenesis.

1) During embryogenesis, I used both the proliferation marker (BrdU, **Chapter 2**) to monitor the mitotic activity of progenitor cells (neuroblasts) and their progeny in building the primary architecture of the embryonic brain, and a specific antibody against the protein expressed by *engrailed* (a segment polarity gene) to identify single neuronal cells to be compared with data available for Insecta (**Chapter 3**). In this way, I obtained information both on the neurobiology of the Marbled crayfish (early development of the brain) and on its phylogeny compare to other arthropods. Adding my results to the existing literature on neurophylogeny I provided support for the Tetraconata hypothesis.

2) During embryogenesis, specific genes involved in brain formation are expressed. According to previous studies on different species, I selected and cloned three genes from the Marbled crayfish that play a significant role during neurogenesis: *Pax6*, *atonal* and *orthodenticle*. Analysis was essentially on primary sequences that showed an elevated conservation, in fully agreement with the literature. These findings represent a base for future studies, once an appropriate protocol for *in situ* hybridization will be available (**Chapter 4**).

3) After embryogenesis, the development of the brain does not stop: in fact, there is a continuous accumulation of new neurons that are ultimately added in the olfactory system. While neuroblasts disappear within the first post-embryonic stages (POI and POII), a life-long turnover of olfactory interneurons is established by a new structure (the neurogenic niche) that shows up on the ventral surface of the brain around hatching. I explored at which stage the niche starts to contribute to brain development and from where it develops (**Chapter 5**).

In order to achieve such goals, I performed different experiments by using diverse

techniques which have been described in a separated chapter (**Chapter 6**, Material and Methods).

By these studies (on embryonic and postembryonic brain development), I investigated the two different mechanisms of neurogenesis in the crayfish brain: neuroblasts for the embryonic brain formation, glia-driven neurogenesis for the post-hatching period, a type of adult neurogenesis that resembles that in vertebrates.

The study of the brain of the Marbled crayfish revealed significant aspects both on its neuroanatomy and development. This information has been used for comparison with other arthropod groups but also with vertebrates and will be a source of information for future investigations, especially in the exciting field of research of stem cells and adult neurogenesis that is now challenging many researchers to unravel the complex mechanisms of our brain.

Chapter 2

Embryogenesis

2.1 INTRODUCTION

2.1.1. Neurogenesis

Neurogenesis is the process of production of new neuronal cells which leads to the development of the central nervous system (CNS). In *Drosophila*, it involves the conversion of a simple uniform sheet-like layer of ectodermal cells into a three dimensional structure made of a variety of cells. During the first stages of embryonic development, neurogenesis is responsible for building the initial architecture of the brain and the ventral nerve cord. Afterwards, neuronal stem cells and their progeny are likely to undergo positional reorganization and, by differentiation and morphogenesis of the stem cell progeny, the transformation into the highly differentiated structure of the mature CNS occurs (Technau et al., 2006).

2.1.2 Neurogenesis in Arthropoda

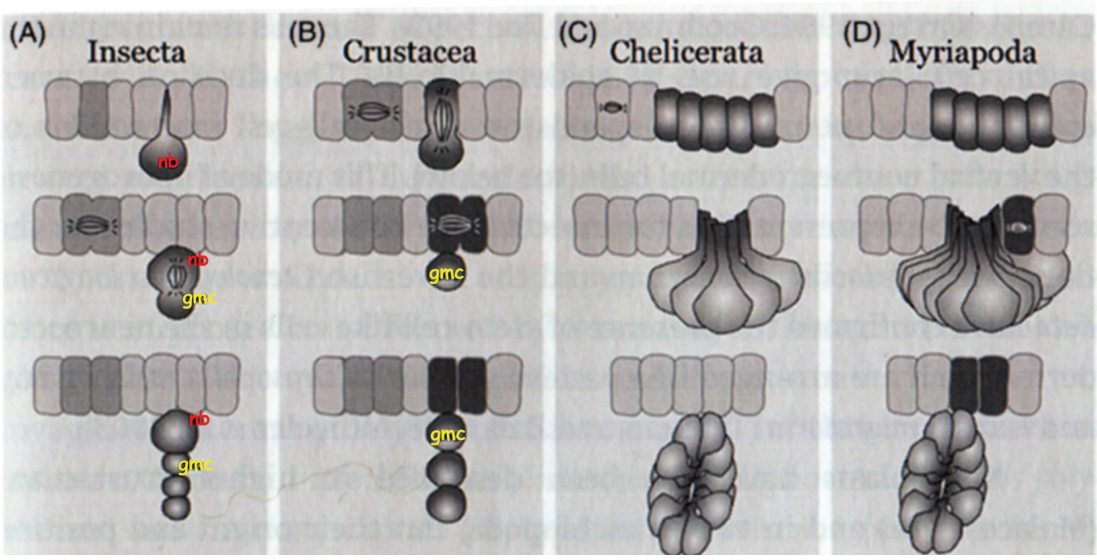


Fig. 2.1- Schematic transverse sections of the embryonic ventral neuroectoderm (light grey. Epidermal cells are in middle grey) of the four arthropod groups during early stages of neurogenesis. The mode by which neurogenesis occurs varies: in insects, neural precursors (multishaded grey) called neuroblasts (nb) delaminate and each divide asymmetrically to produce one ganglion mother cell (gmc) which divides symmetrically to produces two neurons. In crustaceans, neuroblasts remain in the neuroectoderm in the outer layer and apparently divide to produce chains of ganglion mother cells (direct evidence of such division pattern is still missing, a part some recent supporting data on the ventral nerve cord). Crustacean neuroblasts can switch from producing gmc to generating epidermal cells. In both myriapods and chelicerates groups of neural precursors are formed from invagination sites in several waves.In myriapods, in contrast to chelicerates, single mitotic cells (dark grey) and groups of mitotic cells (not shown) are associated with invagination sites (modified from Stollewerk, 2008).

Neurogenesis has been studied in the four main groups of arthropods, especially the ventral nerve cord. Similar and most likely homologous structures, such as the ground pattern of the brain and the main neuromeres as well as sets of homologous neurons, arise during arthropod development, but the modes of generation are different and each group shows its particular development pathway (Fig. 2.1).

2.1.2.1. Neurogenesis in insects: the stem cell mode

Nervous system development has been extensively examined in insects such as *Drosophila melanogaster* (Hartenstein and Campos-Ortega, 1984). The mode of development is via neuroblasts which are specific neuroectodermal cells that repeatedly divide unequally, each giving rise to a smaller ganglion mother cell that is pushed dorsally into the interior of the embryo and divides once more to give rise to neurons or glial cells ("stem cell mode") (Review: Technau et al., 2006). In the ventral neuroectoderm of *Drosophila*, a layer of mixed neural and ectodermal cells is present and fate of these cells depends on the expression of specific proneural genes: *achaete*, *scute*, and *lethal of scute* (Jiménez and Campos-Ortega 1990, Cabrera et al. 1987). In fact, these genes are initially expressed in defined small clusters of cells within the neuroectoderm and, as development proceeds, their expression becomes restricted to a single cell of each cluster, the future neuroblast (Cabrera et al. 1987, Romani et al. 1989, Skeath and Carroll 1992). In each cluster, the unique fate acquired by the neuroblast is determined by its position and time of formation in the neuroectoderm (Campos-Ortega, 1992). This process is called lateral inhibition and is mediated by the neurogenic genes *Notch* and *Delta* (Martin-Bermudo et al. 1995, Seugnet et al. 1997). Each neuroblast has an individual identity and gives rise to an invariant lineage of progeny. For this reason, neuroblasts do not remain in the neuroectoderm (as we will see in crustaceans) because they produce exclusively neural cells (in this context both neurons and glia cells are considered neural cells). Neuroblasts leave the neuroectodermal layer by delaminating into the embryo in five discrete steps and divide asymmetrically to generate ganglion mother cells in a well defined spatio-temporal pattern (Goodman and Doe 1993, Technau et al. 2006) (Fig. 2.1A). Ganglion mother cells divide once to give rise to neural cells that differentiate into neurons and glia. Similar pattern of neuroblasts has been found in other

insects such as *Tribolium castaneum*, *Tenebrio molitor* and *Schistocerca americana* (Bate 1976, Bate and Grunewald 1981, Shepherd and Bate 1990, Urbach et al. 2003). The remaining cells of the neuroectoderm (specialized epidermoblasts, EBs) will generate the ventral epidermis. In the brain, proneural genes are expressed in larger domains, rather than small clusters, and NBs appear to segregate not in waves but continuously (Urbach et al., 2003). In grasshopper, about 100 brain NBs have been identified in each side of the brain, based on the expression of more than 40 molecular markers which represent 34 different genes. Each NB expresses a specific combination of these genes (Doe 1992, Urbach and Technau 2003).

2.1.2.2. Neurogenesis in chelicerates and myriapods: the invagination mode

Less is known about neurogenesis in the other groups of arthropods. In chelicerates and myriapods, no neuroblast has been detected so far and, instead, groups of five to nine neural precursors, bottle like-shape, within the neuroectoderm, form invagination sites that eventually segregate from the apical surface and differentiate into neural cells (Stollewerk et al. 2001, Stollewerk and Chipman 2006) (Fig. 2.1C, D). Similar to *Drosophila*, proneural genes have been identified in spiders, *CsASH1* and *CsASH2*, that are expressed during development of the nervous system (Stollewerk et al., 2001). *CsASH1* is required for the formation of the invagination site and the process of invagination, whereas *CsASH2* is expressed later during neurogenesis in the invaginating cells and apparently is required for cells differentiation.

2.1.2.3. Neurogenesis in crustaceans

Compared to insects, the crustacean neurogenesis is not as well documented yet, especially regarding the embryology of the brain (Fig. 2.1B). The very first stages of embryogenesis, from gastrulation to segment formation, anlagen of limb buds, and early differentiation of ganglia, were analyzed by Scholtz (1992) in the Australian crayfish *Cherax destructo*r. Among Malacostraca, the first embryonic stage is commonly represented by the so called egg-nauplius, an early germ band that resides on a large amount of yolk that is covered by extra-embryonic cells. The most anterior region is the ocular-protocerebral region and the lateral eyes, followed by the segments associated

with the antenna I, antenna II and the mandible and the posterior growth zone which generates the material for the post- naupliar segments. The body is pear-shaped with a broad anterior part bearing a nauplius eye (Scholtz, 2000) (Fig. 2.2). The naupliar segments are formed simultaneously by spotted ectoderm cells arranged in a V-shaped germ disc, anterior to the blastopore (Scholtz, 1992). No specific cell division pattern is recognizable. Afterwards, the post-naupliar segments are formed by the action of ectoteloblasts in the posterior growth zone, from front to back, and the germ band extends and develops through an orderly process in which the pattern of cell divisions is quite stereotyped (Patel et al., 1989).

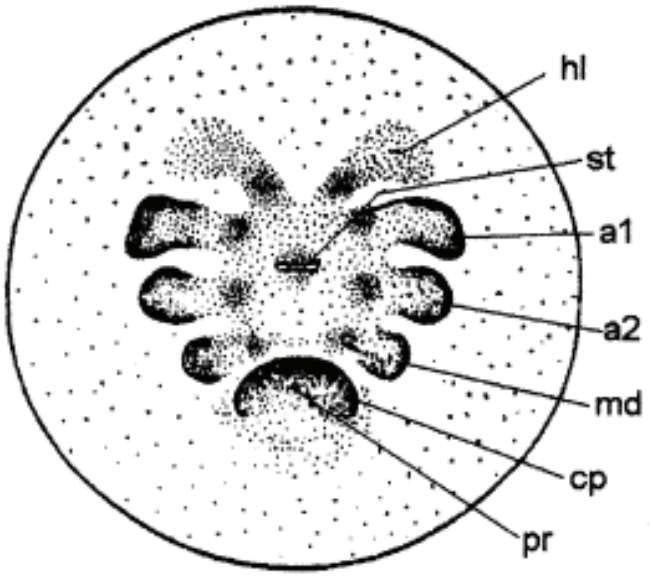


Fig. 2.2- Scheme of a malacostracan egg-naplius. Abbr: hl, head lobe; st, stomodaeum; a1, antenna I; a2, antenna II; md, mandible; cp, caudal papilla; pr, proctodaeum. (From Scholtz, 2000).

The first ectoteloblasts (ETs) can be detected between 20 and 25% of embryonic development (E20-25%, developmental stages by Sandeman and Sandeman, 1991) and differentiate from the blastoderm. Ectoteloblasts are specialized stem cells located in the posterior region of the germ band, anterior to the proctodeum (Scholtz, 1990). At E30%, ectoteloblasts form a complete ring consisting of 39-46 cells (the final number varies between individuals) and most of the post-naupliar material comes from these cells. An inner ring of eight mesoteloblasts (four per side) in the caudal papilla, that will give rise to the mesodermal structure, shows a conserved characteristic arrangement among Malacostraca (Scholtz 1992, 2000) (Fig. 2.3). Once differentiated, the ectoteloblasts divide unequally giving rise to smaller progeny. The ectoteloblasts divide asymmetrically

in synchrony, each round of division gives rise to a row of smaller ectoteloblast progeny. With each round of division, the ectoteloblasts are displaced caudally, leaving behind rows of progeny (Fig. 2.4). These progeny cells are arranged in precise longitudinal and transverse rows (15) which create a grid-like pattern. Segmental structures (limbs, ganglia) are composed of derivatives of 2 ectoderm rows.

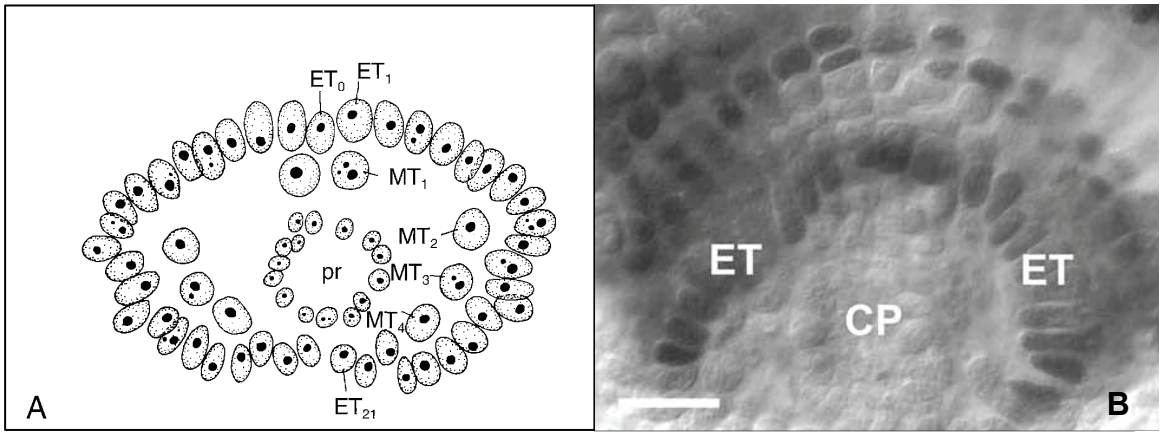


Fig. 2.3- A: ring of teloblasts in an embryo of *C. destructor* at E35% (dorsal is towards the top, ventral towards the bottom). The teloblast ring surrounds the proctodaeum (pr; from Scholtz 1992). B: Transverse section through the En+(Engrailed positive)-teloblast rings of the Marbled crayfish. CP: caudal papilla (En: Engrailed, from Fabritius-Vilpoux et al., 2008). Scale bar: 10µm.

The ET progeny undergoes a stereotypic pattern of differential cleavage by which neuroblasts are formed. The first two neuroblasts differentiate during the forth differential cleavage of a specific row at about 35% (Fig. 2.5). They can be identified by their typical mode of division that produces columns of ganglion mother cells toward the centre of the embryo, beneath the outer ectoderm layer, deeper within the yolk. Ganglion mother cells, in turn, divide equally giving rise to neurons (Scholtz, 1992). As development continues, the number of cells increases and segmentation and limb formation take place, so that appearance of neuroblasts and analysis of division pattern becomes more difficult.

In the freshwater crayfish *Cherax destructor*, thoracic neuroblasts are arranged in a regular bilateral pattern of several files and columns plus an additional median neuroblast and a total of 25–30 neuroblasts per hemineuromere has been reported (Scholtz, 1992). Harzsch (2001), by using the s-phase proliferation marker bromodeoxyuridine (BrdU) to examine neuroblast proliferation during early neurogenesis in embryos of the shrimp

Palaemonetes argentinus, confirmed the general results of Scholtz (1992) and reported 14–18 neuroblasts to be present in each hemineuromere at E35% (Fig. 2.4).

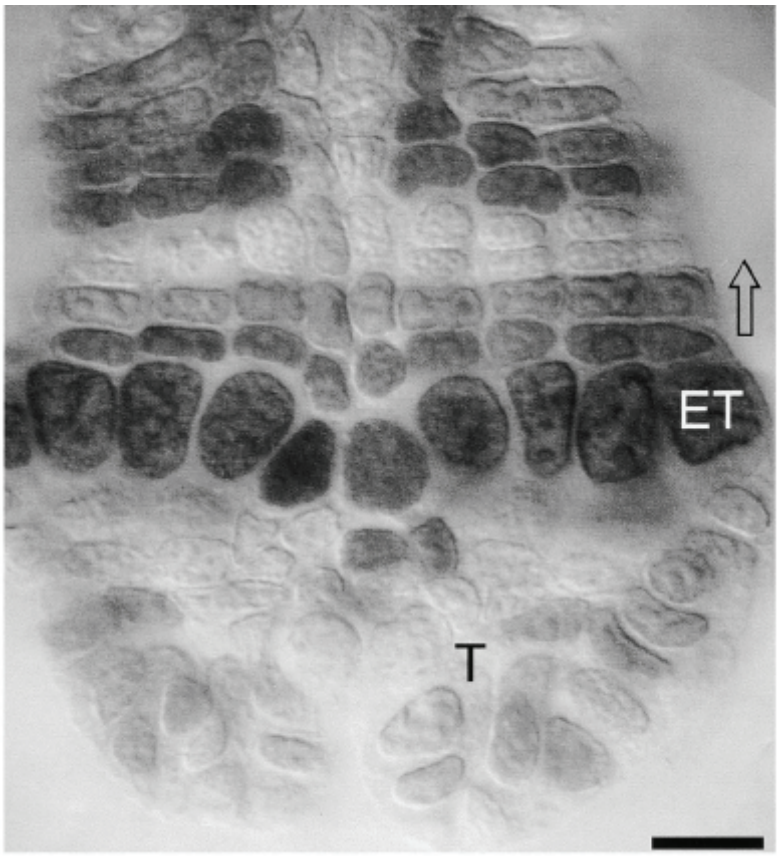


Fig. 2.4- Caudal papilla of an embryo of *Palaemonetes argentinus* at E30% after 4 h incubation in BrdU and subsequent immunohistochemical detection. The arrow indicates the direction into which the ectoteloblast (ET) within the caudal growth zone (located in front of the telson T) proliferate new segments. ET: Ectoteloblasts; T: Telson. Scale bar:15µm (image and text from Harzsch, 2001).

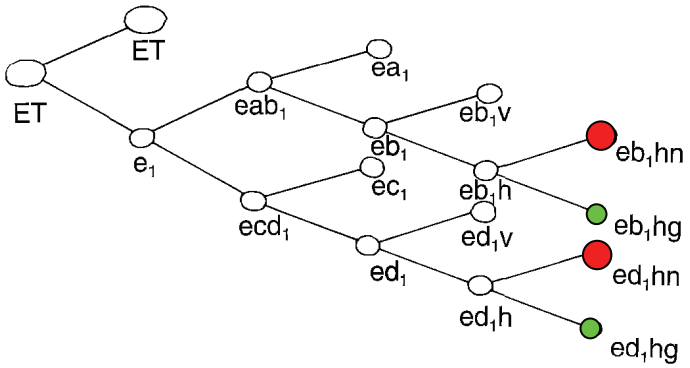


Fig. 2.5- Schematic representation of the lineage of the first neuroblasts and ganglion mother cells in the thoracomeres of *C. destructor* embryos (lettering in lower case identifies individual cells). ET: ectoteloblasts. The first two neuroblasts are generated during differential cleavage of row e. The divisions of cells b_1h and d_1h are unequal, the larger division products, the neuroblasts $b_1$hn and $d_1$hn (red cells), remain in the surface layer. Their sister cells, the ganglion mother cells $b_1$hg and $d_1$hg (green cells) lie deeper within the yolk (image and text from Scholtz 1992).

The ventral nerve cord of embryos and larvae of selected decapod crustaceans was examined by BrdU labeling also by Harzsch and Dawirs (1994), Harzsch (2001), by phalloidin-staining which labels actin (Vilpoux et al., 2006), histamine (Rieger et Harzsch, 2007) and by engrailed immunodetection (Fabritius-Vilpoux et al., 2008).
Development of the embryo proceeds (Fig. 2.6). During neurogenesis, NBs remain at the embryo surface amalgamated with ectoderm cells. NBs arise continuously in the two initial cells columns close to the midline, while the more lateral cells form limbs (Scholtz 1990, 1992, Ungerer and Scholtz 2008). As already mentioned, and contrary to insects, crustacean neuroblasts do not delaminate into the embryo but remain in the outer surface and they can generate epidermal cells after budding off ganglion mother cells (Scholtz and Gerberding, 2002). Another difference compare to insects is that, in malacostracans, NBs originate from ectoteloblasts by an invariant lineage, whereas NBs in insects differentiate without a defined lineage by cell-to-cell interactions within the neuroectoderm. Moreover, some NBs survive the early postembryonic period of neurogenesis (POI in the Marbled crayfish) and may contribute to the process of adult neurogenesis, an important and vital process of generating new olfactory neurons during adulthood. The chapter 5 will deal with this subject.

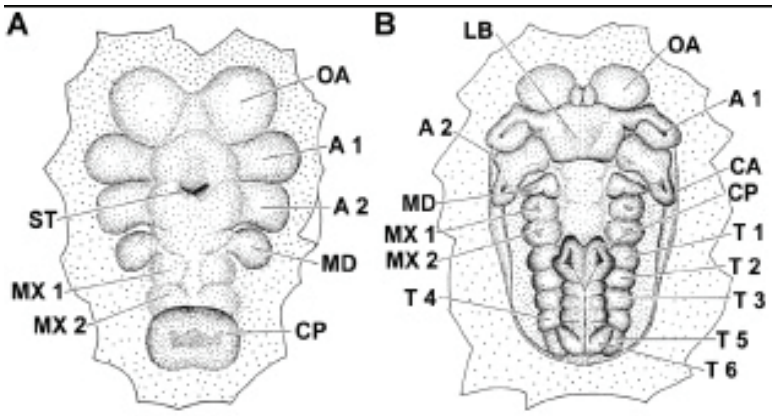


Fig. 2.6- A, B: Embryos of the crayfish *Cherax destructor* at E35% (A) and E45% (B).. Abbr.: A 1, 2: antenna one and two, CA: carapace, CP: caudal papilla, ET: ectoteloblasts, LB: labrum, MD: mandible, MT: mesoteloblasts, MX 1, 2: maxillae one and two, OA: optic anlagen, pr: proctodaeum, ST: stomodaeum, T 1-6: anlagen of thoracic appendages one to six (from Sandeman and Sandeman 1991).

The spatial and temporal patterns of neuroblast proliferation in the ventral nerve cord of late embryos and larvae of selected decapod crustaceans were examined by BrdU. Regarding the dynamics of neurogenesis, a complete picture of this process is not available yet. In the species investigated so far, from the appearance of first neuroblasts to the end of embryogenesis, it has been shown that the number of active neuroblasts can

reach up to 30 per hemiganglion (Scholtz, 1992). It then decreases until a small subset of neuroblasts remain active prior end of the embryo life. Using the BrdU technique, Sullivan and MacMillan (2001) showed that in later embryonic stages of *Cherax destructor* fewer neuroblasts are present than Scholtz (1992) encountered in the early embryos. The number of neuroblasts is also smaller in late embryos of the American lobster and the spider crab (Harzsch et al., 1998) and spider crab larvae (Harzsch and Dawirs, 1994). This signifies that the number of neuroblasts may vary among decapod species and is dynamic throughout embryogenesis.

Contrary to insects in which the complete lineage of several NBs up to the formation of neurons is known, identification of crustaceans NBs by histological or molecular means have not been achieved so far. A recent study by Ungerer and Scholtz (2008) accomplished this goal by labeling *in vivo* with a fluorescent dye individual NBs in the ventral nerve cord of the amphipod *Orchestia cavimana*. A partial neuroblast map of single NBs and their lineage to neurons was obtained and compared to that of insects, revealing strong homology that supports once again the Tetraconata hypothesis (Fig. 2.7).

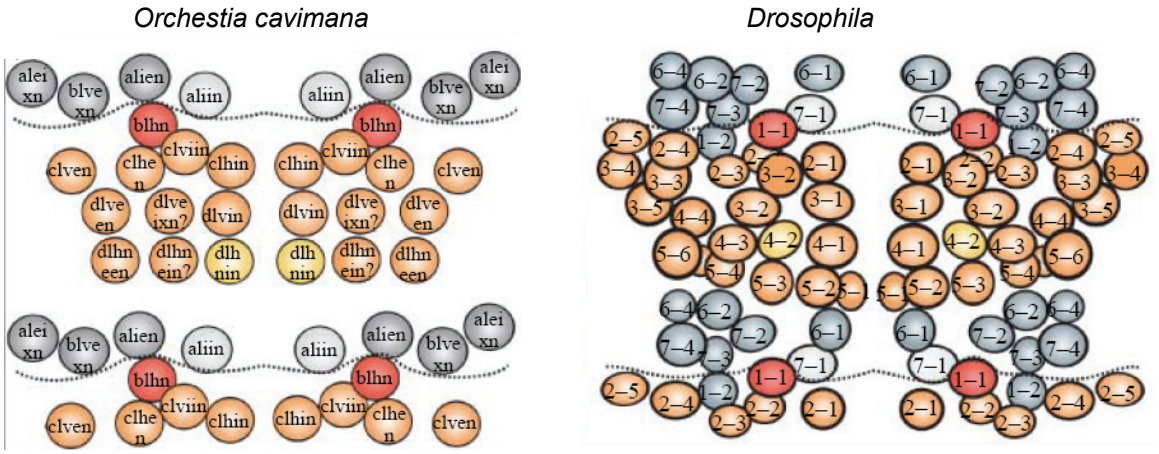


Fig. 2.7- Comparison between neuroblast map of thoracic segment in *Orchestia* and *Drosophila*. b1hn (homologous to 1–1 in insects) is marked red. Yellow and light grey NBs are supposed to have homologous counterparts in insects. Greyshaded NBs are engrailed positive (from Ungerer and Scholtz, 2008).

2.1.3. Neurogenesis in the brain

Vilpoux and collegues (2006) analyzed the embryonic development of the central nervous system in the Australian crayfish and the Marbled crayfish by using phalloidin and an anti-synapsin anti-serum, both good markers for the developing CNS: phalloidin

is a probe for actin which is enriched in the axonal tracts while synapsin is specific for synaptic proteins and can be used in later embryos where the musculature (rich in actin as well and thus reactive to phalloidin) could mislead the interpretation of results. This study showed the formation of the primordial scaffold of the CNS in crayfish embryos. As seen above (from Scholtz 2000, Sandeman and Sandeman 1991. Fig. 2.2, 2.6), in the egg-nauplius the anterior part of the embryo (the primitive brain) comprises paired optic anlagen, paired antenna I and II anlagen and the paired mandible anlagen. The naupliar proto-, deuto- and trito cerebrum and the mandible neuromere emerge simultaneously in the naupliar brain. These four neuromeres were suggested to be arranged in mirror symmetry with respect to an imaginary transverse line across the stomodeum (Vilpoux et al., 2006). In the nauplius stage, the primordial brain is arranged in an orthogonal pattern, and forms a ring around the stomodeum (Fig. 2.8).

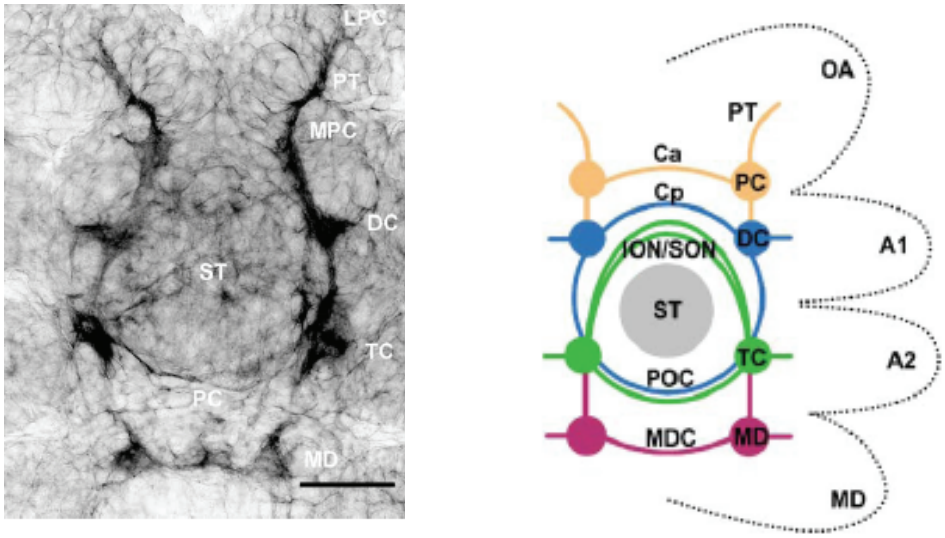


Fig. 2.8- On the left: brain of the Marmorkrebs at E39% stained with phalloidin that shows the early scaffold of the naupliar brain, which is the first sign of the central nervous system. Scale bars: 50 µm. On the right: Schematic, idealised model of the naupliar brain. The dotted lines on the right depict the outlines of the egg-nauplius. A1, A2 Antenna one and two; DC deutocerebrum; PC protocerebrum; LPC lateral protocerebrum; MD mandible; MPC median protocerebrum; OA optic anlagen; PC/POC post-oesophageal commissure; PT protocerebral tract; ST stomodeum; TC tritocerebrum.Ca, Cp anterior and posterior pre-oesophageal commissures; ION inferior oesophageal nerve; MDC mandibular commissure; SON superior oesophageal nerve (from Vilpoux et al., 2006).

In coincidence with the emergence of the early scaffold of the brain, neuropils in the eyestalk anlagen are established at around E35%, together with the first sign of

hemiellipsoid body and the medulla terminalis that will increase in size by E60%. Between E46% and 60% the ganglion anlagen of all segments are laid down (Vilpoux et al., 2006). The nerves from the deuto- and trito-cerebrum enter the antenna I and II in embryos at E60%. Meanwhile, in the anterior region of the embryo, the labrum is formed at the anterior margin of the stomodaeum. With further development, the labrum grows posteriorly and covers the mouth (Scholtz, 1992).

The embryonic brain of Crustacea has been also analyzed by antibody against Engrailed (Patel et al. 1989, Scholtz 1995a, Sintoni et al. 2007 see Chapter 3) and by using BrdU to monitor the mitotic activity of neuroblasts (Fig. 2.9). In fact, neuroblasts are obviously essential for the formation of the decapod brain (Harzsch and Dawirs 1996a, Harzsch et al. 1999b) and visual system (Harzsch and Dawirs 1995a, 1996a, Harzsch et al. 1999a).

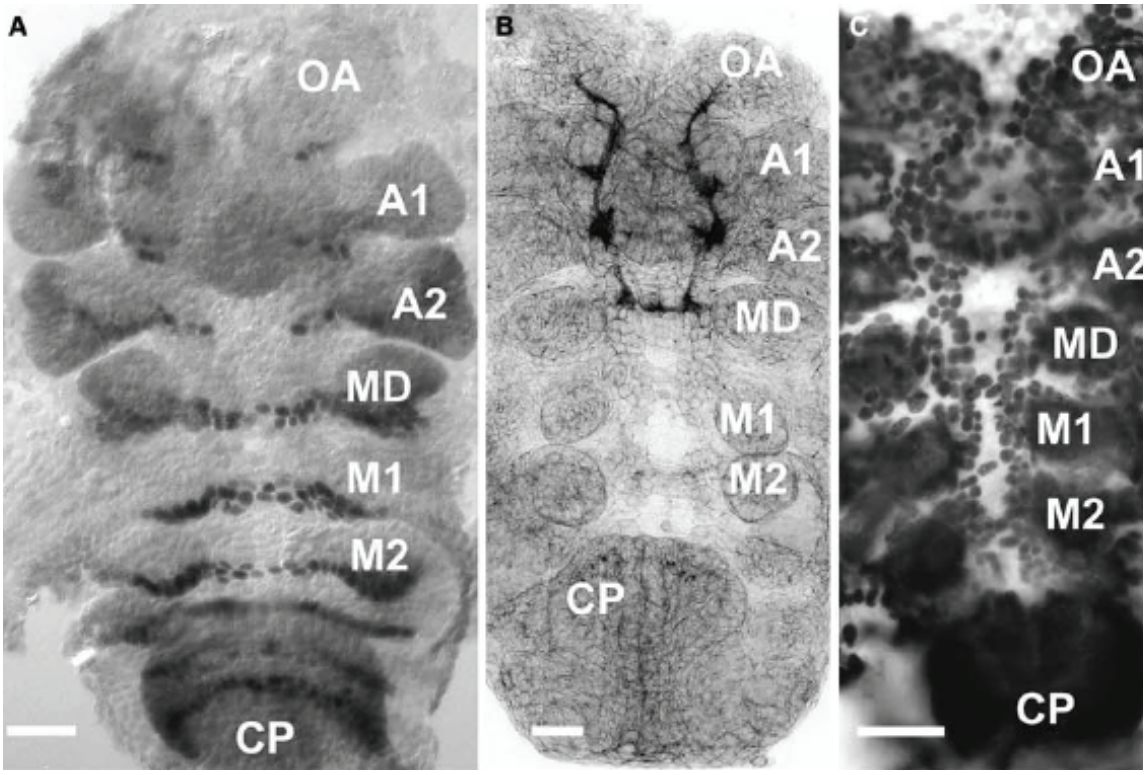


Fig. 2.9- Comparison of embryo- and neuro-genesis analyzed by using anti-Engrailed (A), phalloidin (B) and BrdU (C), in the Marbled crayfish (Fabritius-Vilpoux et al., 2008). Whole mounts of dissected E38% embryos, ventral views. C: Labelling with the mitosis marker bromodeoxyuridine reveals a high level of mitotic activity throughout the entire embryo including the brain, limb anlagen and the developing ventral nerve cord. Abbreviations: A1, A2 Antenna one and two; CP caudal papilla; ET ectoteloblast; MD mandible segment; M1, M2 maxilla one and two segments; OA optic anlagen. Scale bars a–c 50 μm.

2.1.4. "A common plan for neuronal development"

This was the suggestion of Thomas et al (1984) regarding Insecta and malacostracan Crustacea, based on specific neurons (pioneer neurons) that were responsible for pioneering major axon pathways in the embryonic CNS of both insects and crustaceans. More studies identified neurons, axon morphology and timing of axonal outgrowth confirming the existence of a set of homologous pioneer neurons in insects and crustaceans that finds no counterpart in myriapods (see Ungerer and Scholtz 2008, Whitington et al. 1991, 1993). Moreover, Gerberding and Scholtz (1999, 2001) identified a midline NB that shows similarities to homologous cells in insects. One of the morphological characters that support a monophyletic Crustacea-Insecta unit is in fact the mode of development of the central nervous system *via* neuroblasts. However, as mentioned before, there are differences between crustaceans and insects NBs and actually, so far, the only common feature of neurogenesis between Malacostraca and Insecta is the presence of neuronal stem cells that divide asymmetrically. Homology of cells such as neuroblasts or neurons can only be proven when it can be shown that these cells share an identical lineage (Doe 1992, Boyan and Williams 1995). So, homology of stem cells and the hypothesis brought up by Thomas et al (1984) could not be proven yet, although recent data provide strong support (Ungerer and Scholtz, 2008). Molecular profiles can provide support to specify the identity of NBs as well. So far, only in *Drosophila* a detailed picture of gene expression has been provided. Segment polarity genes have been identified in other insects, in crustaceans, in myriapods, and in a spider, but except for Engrailed (Chapter 3) their expressions during neurogenesis have not been studied. In the project described in Chapter 4, three genes involved in neurogenesis (*Pax6*, *atonal* and *orthodenticle*) have been isolated from the Marbled crayfish and, in future, by establishing an appropriate *in situ* hybridization protocol, it would be possible to identify specific NBs expressing them.

2.1.5. Neurogenesis in the brain of the Marbled crayfish

The detailed analysis and temporal sequence of stem cells proliferation in the early embryonic brain of crustaceans has never been reported so far. As said above, the majority of studies have focused in the ventral nerve cord of diverse species, either very

early stages (like cleavage and segmentation) or late embryonic development. Here, I provided the cell analysis of neuroblasts and their associated progeny from the egg-naplius stage (around E38%) of the Marbled crayfish to later embryonic development (around 70%).

The development of the brain from 70% until juveniles (post-embryonic stage) will be described in the Chapter 5.

Results are based on the use of the 5-Bromo-2-deoxyuridine (BrdU), a synthetic thymidine analog, which is incorporated into single strand DNA during the S-phase of the cell cycle.

2.2 RESULTS

2.2.1. Neurogenesis in early development

Development of the freshwater Marbled crayfish can be easily followed from the egg-nauplius-stage by relying on the use of the BrdU staining of S-phase cells (about E35%, based on staging system of Seitz et al., 2005) (Plate, Pl., 2.1). As described by Scholtz (1992, 2000. Pl. 2.1, inset), the egg-nauplius shows an anterior protocerebral-ocular region which will give rise to the lateral compound eyes (optic lobes), anlagen of antenna I, antenna II and mandible, and a posterior part represented by the caudal papilla (Pl. 2.1, 2.2A). In the naupliar brain, the optic lobes are not well defined. BrdU+ cells (BrdU positive-cells) that participate to the formation of the eyes are not yet assembled to form a specific structure, but they appear to be diffusely distributed and continuous with the thin embryonic tissue that surrounds the germ disc. The mandible is not clearly visible either. It can be said that, at this stage, except for the antennae, the egg-nauplius resembles a group of cells which is about to organize within a more compact and defined structure, the forming embryo, but with not a precise structure yet. Antenna I, antenna II and the mandible appear uniramous although, later on in development, antenna II will become bilobed. The typical regular arrangement of ectoteloblasts which will form a ring around the caudal papilla later during development is not yet discernible (Pl. 2.1, 2.2A). In slightly older embryos (E36%, Pl. 2A), the typical regular grid-like pattern arrangement of ectoteloblasts and their progeny in the forming ventral nerve cord is more evident, only a more compact layer of dividing cells delineate the embryo from the contiguous developing tissue. At E38%, the optic lobes still are not clearly delineated from the surrounding cellular tissue (Pl. 2B). Antenna I and II and the mandible are more distinct, in particular antenna II starts to enlarge laterally, in the process of becoming biramous later on. The two pairs of maxillae appear as limb buds. The ectoteloblasts ring surrounds the proctodeum in the caudal papilla. During the first stages of neurogenesis, one single median neuroblast appears in the ventral neuroectoderm of the mandible neuromere (Pl. 2.3). The bromodeoxyuridine labeling shows an intense mitotic activity throughout the entire embryo including the brain and the developing ventral nerve cord (Pl. 2.1, 2.2, 2.3).

As the development of the embryo proceeds, new segments are added in the caudal growth zone by the mitotic action of ectoteloblasts in an anterior–posterior gradient (Pl. 2.4). By E42%, both maxillae (maxilla I and II) appear distinctly, the antenna X becomes bilobed and one median neuroblast appears in each ventral neuromere. The BrdU staining reveals a high mitotic activity in the brain, limbs buds and caudal papilla (Pl.2.4).

At 46% of development, optic lobes, antenna I, antenna II, mandible, maxilla I and II are well defined (Pl. 2.5). Thoracic segments are forming and appear longitudinally elongated. The caudal papilla is now ventrally/anteriorly flexed, thus obscuring the ventral nerve cord (Pl. 2.5A). The phalloidin staining, which stains actin that is enriched in the axonal tracts, reveals the axonal architecture of the embryonic brain at the stage of 44-46% (Pl. 2.5B). Neurons have already extended neurites in the developing nervous system and the embryonic brain scaffold looks already subdivided into the three neuromeres: the protocerebrum, deutocerebrum and tritocerebrum. In the protocerebral-ocular region, the hemiellipsoid bodies are highlighted as well. A pair of fibrous parallel commisures, called post-oesophageal commisure (PC) and mandibular commisure (MC), links the paired tritocerebral and mandibular neuromeres (Pl. 2.5B).

In Pl. 2.6, brains of different developmental stage, from early differentiation (E36%) to later (E50%), are shown. As explained above, main modifications occur at the level of the appendages, such as ocular-protocerebral lobes, antenna I, II and mandible. The size of the brain increases as well as the entire embryonic body. From a thin layer of mitotic cells, the brain goes through a series of morphogenetic rearrangements and cell relocation that define, as development proceeds, the main compartments and the tridimensional structure of the brain. Neuroblasts, in fact, from the ventral neuroectoderm are supposed to divide and give rise to ganglion mother cells that presumably are displaced toward the centre of the embryo, and thus underneath the ectoderm layer (Scholtz 1992, 2000, Harzsch and Dawirs 1994, Harzsch et al. 1998, Gerberding and Scholtz 1999, 2001, Sullivan and MacMillan 2001). In earlier stages (Pl. 2.6A-B), no recognizable stereotypic arrangement is detectable in any part of the brain. The BrdU positive cells (BrdU+) are more or less homogeneously distributed in the brain, with more pronounced accumulations at the distal parts of the appendages (Antenna I and II). The optic lobes, characterized by an initial uniform distribution, start to differentiate as demonstrated by

the different arrangement that BrdU+ cells acquire as development proceeds (Pl. 2.6, 2.9B, D). Development of the optical lobes in the Marbled crayfish is a topic that has not be covered in details by the current project. However, it can be said that, in agreement with the literature (Elofsson 1969, Harzsch et al. 1999a, Harzsch 2002b), *in vivo* incorporation of BrdU revealed the presence of distinct proliferation zones that are arranged in semicircle (see Pl. 5.2A, B, Chapter 5).

The central part of the brain, where the stomodeum appears as a circular aggregate of cells, shows a homogeneously layer of cells in early stages (Pl. 2.6A, B). Later on, the central region divides into two halves, each of them characterized by three main groups of cells that represent the proto-, deuto-, and trito-cerebrum (Pl. 2.6E, F).

By E38-40% (Pl. 2.6C, D), the paired deutocerebral region shows a pattern of cells that will be recalled throughout the entire embryonic development and that, in the present study, has already been identified as the origin of a later proliferative region. These bilaterally paired proliferative regions are composed of six to seven cells, with a spherical nucleus, and arranged in circles. I named them “rosetta” and, as it will be explained in fully details in Chapter 5, they are thought to be involved in the formation of the Deutocerebral Proliferative System. These paired clusters of cells will give rise to the proliferative zone in cluster 10 (distinct cluster of projection neurons located lateral to the olfactory lobe) and, in each ventral side of the deutocerebral regions of the brain, to the *neurogenic niche*, a post-embryonic structure responsible for adult neurogenesis in Crustacea (see Chapter 5). The neuroblasts are regularly labeled by BrdU and, thus, they show a relatively short cell cycle time and a non-stop activity because the “rosetta” pattern appears on the ventral side of every embryo of all following developmental stages, until hatching (Pl. 2.6, 2.9).

An overview of the neuroblasts and their associated progeny is provided in Pl. 2.7. Neuroblasts can be recognized by the bigger size and spherical shape of the nuclei that likely resembles that of the cell. The progeny, most likely represented by ganglion mother cells and neurons, are smaller and are tightly associated, and often surrounding the “mother” cell (Pl. 2.7A-F). In some other cases, the relation between cells is not so clear. Cells appear not so close to each others or they show more or less the same size and this makes difficult distinguishing the identity of each cell (Pl. 2.7D, G, H). For

instance, in Pl. 2.7D a typical "rosetta" is shown in which six bigger cells are arranged in circle, and no progeny can be distinguished clearly. To resume, a schematic diagram of the brain and map of precursor cells, adapted from a *camera lucida* drawing, is illustrated in Pl. 2.8A. No depth coding is given, the map represents only the cells which have been usually detected in many specimens in early stages (E40-E44%). The main feature shared by all the examined embryos is the presence of the "rosetta", the proliferative cluster placed in the deutocerebrum (Pl. 2.8B-D).

2.2.2. Neurogenesis in later development

As development proceeds, the embryo increases in size and antenna I and II are more developed (Pl. 2.9A, B). All segments are present and further development is more about growth of appendages. By E55%, the optic anlagen extend laterally, the embryo grows further and the yolk supply (not visible in these specimens) decreases (Pl. 2.9C, D). By actin staining it is possible to observe the optic neuropils that have become more distinct, the caudal papilla that now extends as far as the first maxilla, and in the ventral nerve cord ganglia anlagen appear as pairs of spots on both side of the midline (Pl. 2.10B), transversely connected by a pair of commisures, called anterior and posterior commisures (Pl. 2.10A, B). Neuroblasts keep on dividing, the brain is now clearly divided in two halves and the deutocerebrum appears separated by the protocerebrum above. The cluster of BrdU+ cells (rosetta) that characterized the deutocerebrum defines what, later on in development, will be called the Deutocerebrum Proliferative System (Pl. 2.10C). At this stage, a stereotypic, symmetrical arrangement of cells can be noticed. Both in the optic lobes, proto- and deutocerebrum cells in one half of the brain occupy positions that find their counterpart in the other half of the brain (Pl. 2.10C). This pattern is, to some extent, visible also in earlier embryos (E46-50%. Pl. 2.6E, F) but in later specimens, when NBs starts to decrease in number and the brain is enlarged in size, cells are arranged in an undeniable symmetrical map. A schematic diagram of Pl. 2.10C adapted from a *camera lucida* drawing is given in Pl. 2.11. The right half of the brain show cells arranged according to a depth coding, from ventral to dorsal side. Neuroblasts appear to be situated on the ventral side, whereas their progeny (either ganglion mother cells or neurons) reside more dorsally. Some NBs, in the optic lobes, are associated with only two ganglion

daughter cells that appear to have just budded off. Some others, in the deutocerebral proliferative system, appear circularly surrounded by numerous progeny represented by smaller cells (Pl. 2.11). Concerning the enlargement of the brain and the acquisition of a more complex structure, a comparison between early and late embryo is provided in Pl. 2.12. To understand whether the number of neuroblasts and neurons decreases or increases during development and which size-classes of cells are present in the brain during embryogenesis, a quantitative study was performed. BrdU positive cells of single specimens at a certain developmental stage were counted, the area of the nucleus was measured and plotted in a histogram (Pl. 2.13). In early stages (Pl. 2.13A, B) medium and big cells (more than 150μm^2 area of the nucleus) are dominant, and the mitotic activity is very intense. No small cells (20-50μm^2) are apparently present. From E45% (Pl. 2.13C), smaller cells (from 20 to 50μm^2) represent the highest percentage of cells (about 25%) in the brain and the number of NBs starts to decrease. The situation remains stable until later embryos in which cells of medium size (50-150μm^2) increases, together with the number of smaller cells (from 20 to 85-90%) (Pl. 2.13E, F).

2.2.3. Plates

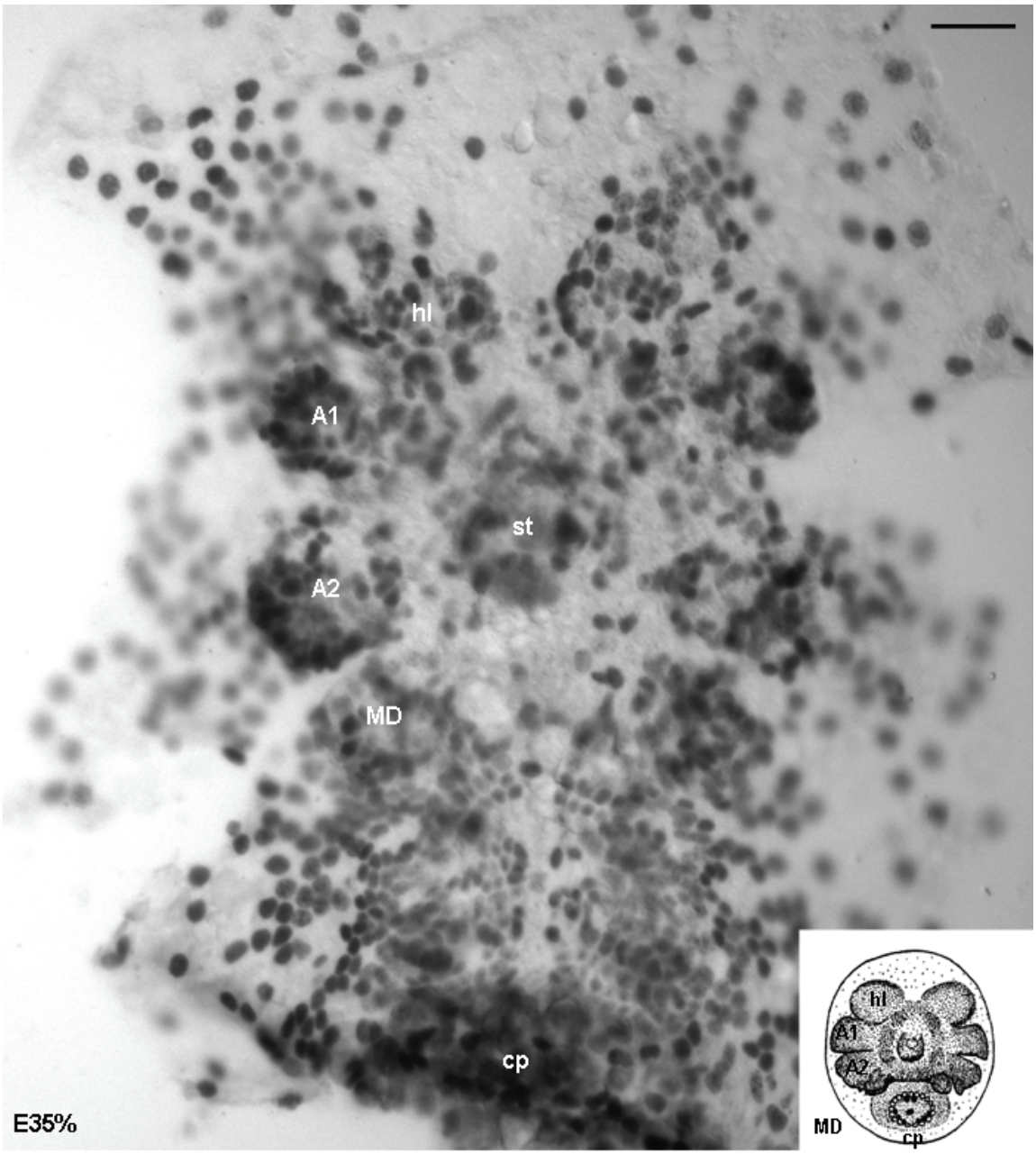


Plate 2.1- The egg-nauplius of the freshwater Marbled crayfish (*Procambarus* sp.). BrdU staining. E35%. In this stage, the anterior region is made of paired head lobes which will give rise to optic lobes, antenna I, antenna II and mandible. The typical regular arrangement of ectoteloblasts which forms a ring around the caudal papilla is not visible. Abbr: hl, head lobe; A1, antenna I; A2, antenna II; MD, mandible; st, stomodeum; cp, caudal papilla. In the inset, schematic drawing of the typical nauplius-egg of Malacostraca (modified from Scholtz, 2000). Scale bar: 50µm.

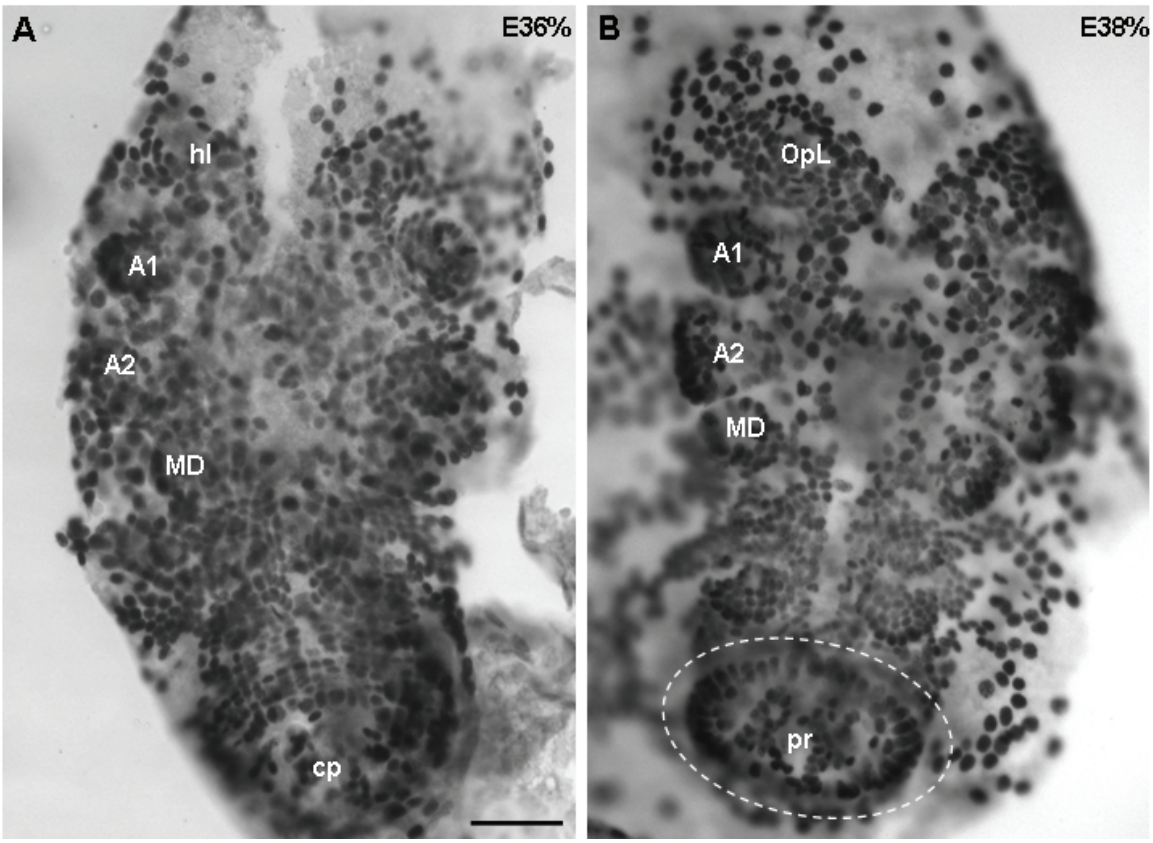


Plate 2.2- The egg-nauplius of the freshwater Marbled crayfish (*Procambarus* sp.). BrdU staining. A: E36%. The anterior region is made of paired head lobes which will give rise to the optic lobes, antenna I, antenna II and mandible. The typical regular grid-like pattern arrangement of ectoteloblasts (white arrow) and their progeny is visible in the forming ventral nerve cord. B: E38%. The ectoteloblasts ring (white dotted circle) surrounds the proctodeum in the caudal papilla. Abbr: hl, head lobe; A1, antenna I; A2, antenna II; MD, mandible; cp, caudal papilla; pr, proctodeum, VNC, ventral nerve cord; OpL, optic lobes. Scale bar: 50µm.

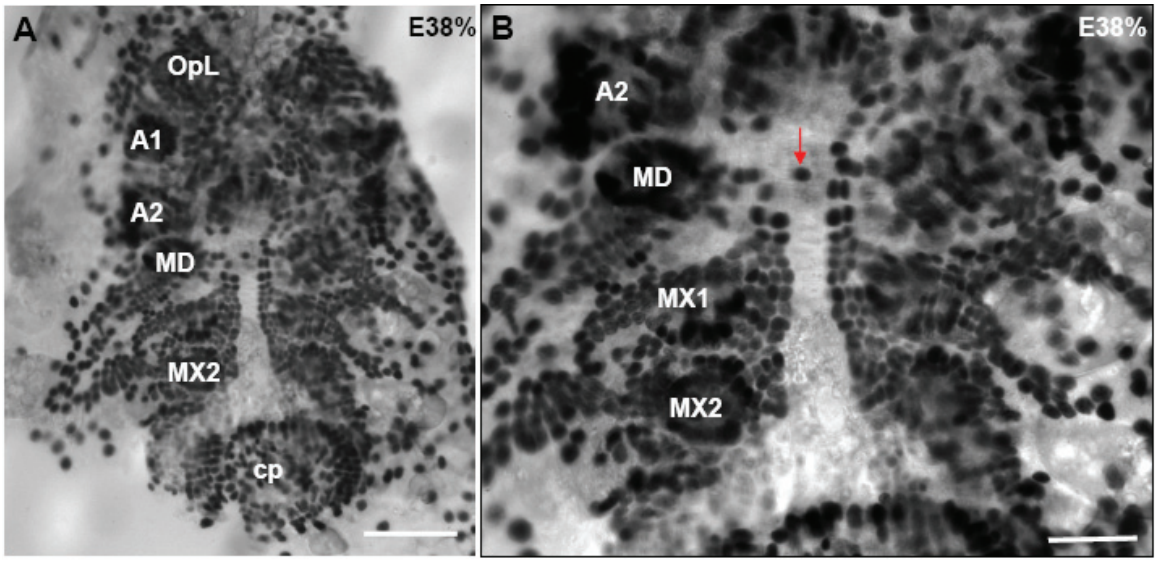


Plate 2.3- The egg-nauplius of the freshwater Marbled crayfish (*Procambarus* sp.). BrdU staining. A: E38%. Scale bar: 50µm. B: higher magnification of the forming ventral nerve cord. The median neuroblast is indicated by the red arrow. Scale bar: 30µm. Abbr: OpL, optic lobes; A1, antenna I; A2, antenna II; MD, mandible; cp, caudal papilla; VNC, ventral nerve cord; MX1, maxilla I; MX2, maxilla II. Scale bar: 50µm.

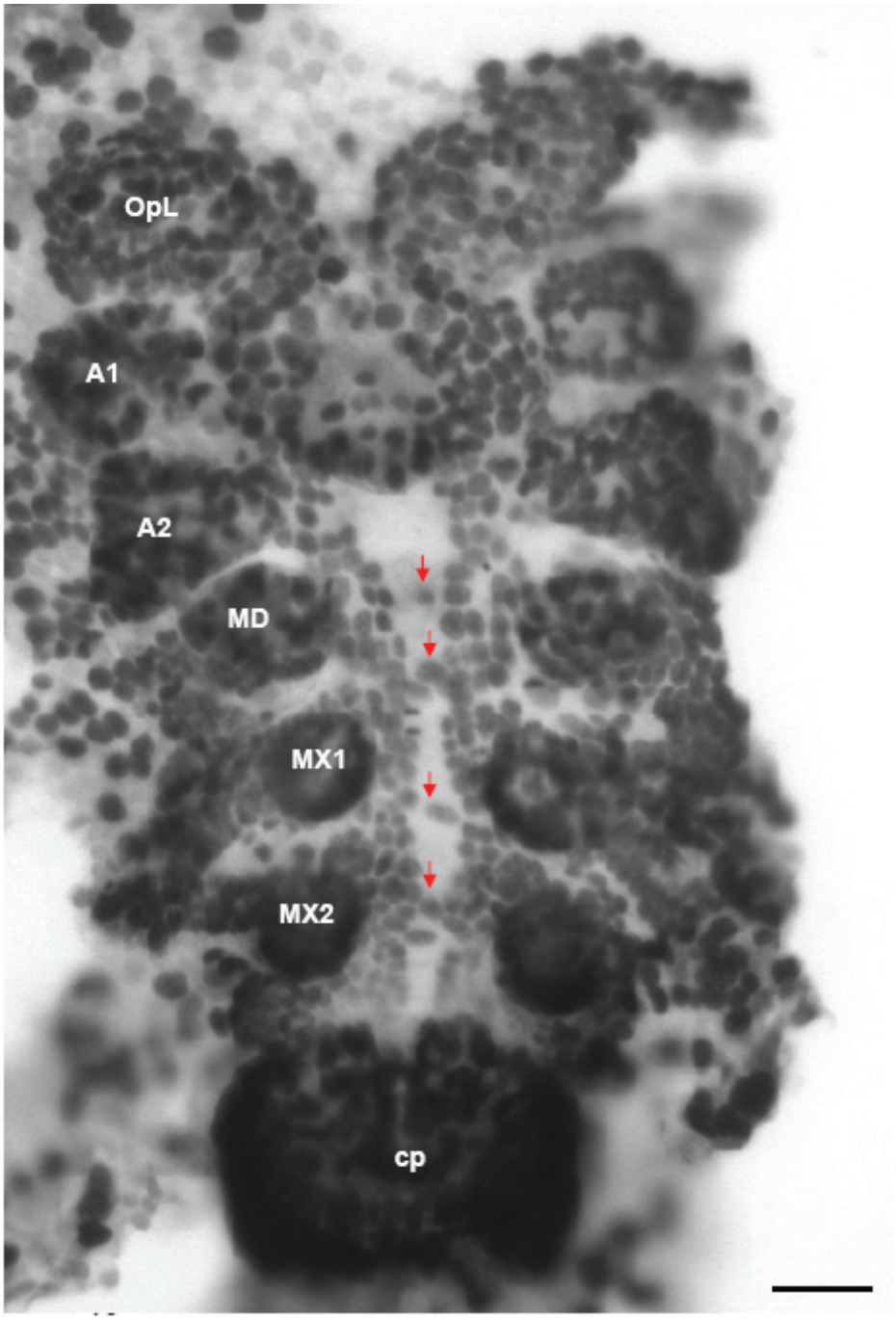


Plate 2.4- Embryo of the freshwater Marbled crayfish (*Procambarus* sp.). BrdU staining. E40%. One median neuroblast appears in each neuromere (red arrow). Abbr: OpL, optic lobes; A1, antenna I; A2, antenna II; MD, mandible; MX1, maxilla I; MX2, maxilla II; cp, caudal papilla. Scale bar: 50µm.

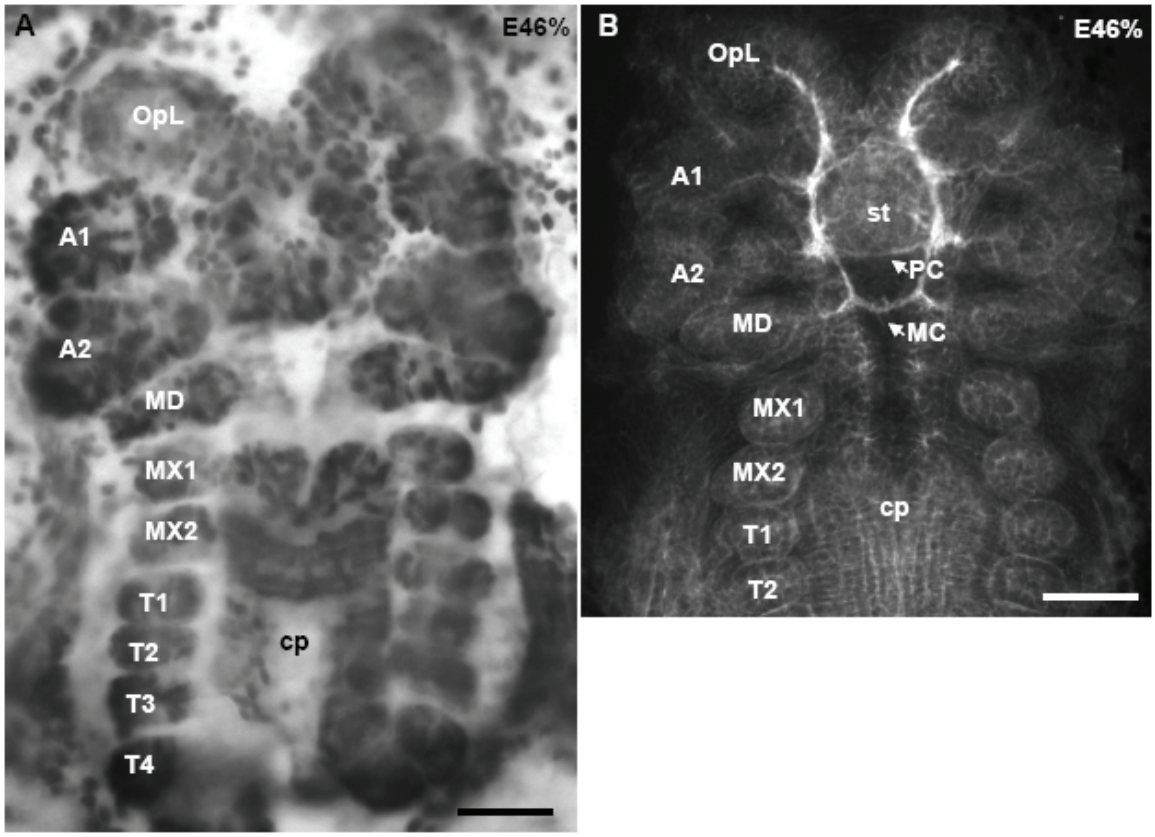


Plate 2.5- Embryo of the freshwater Marbled crayfish (*Procambarus* sp.). A: BrdU staining. E46%. B: phalloidin staining. Abbr: OpL, optic lobes; A1, antenna I; A2, antenna II; MD, mandible; MX1, maxilla I; MX2, maxilla II; cp, caudal papilla; st, stomodeum. PC, post-oesophageal commisure; MC mandibular commisure; T1-4, thoracic segments. Scale bar: 50µm.

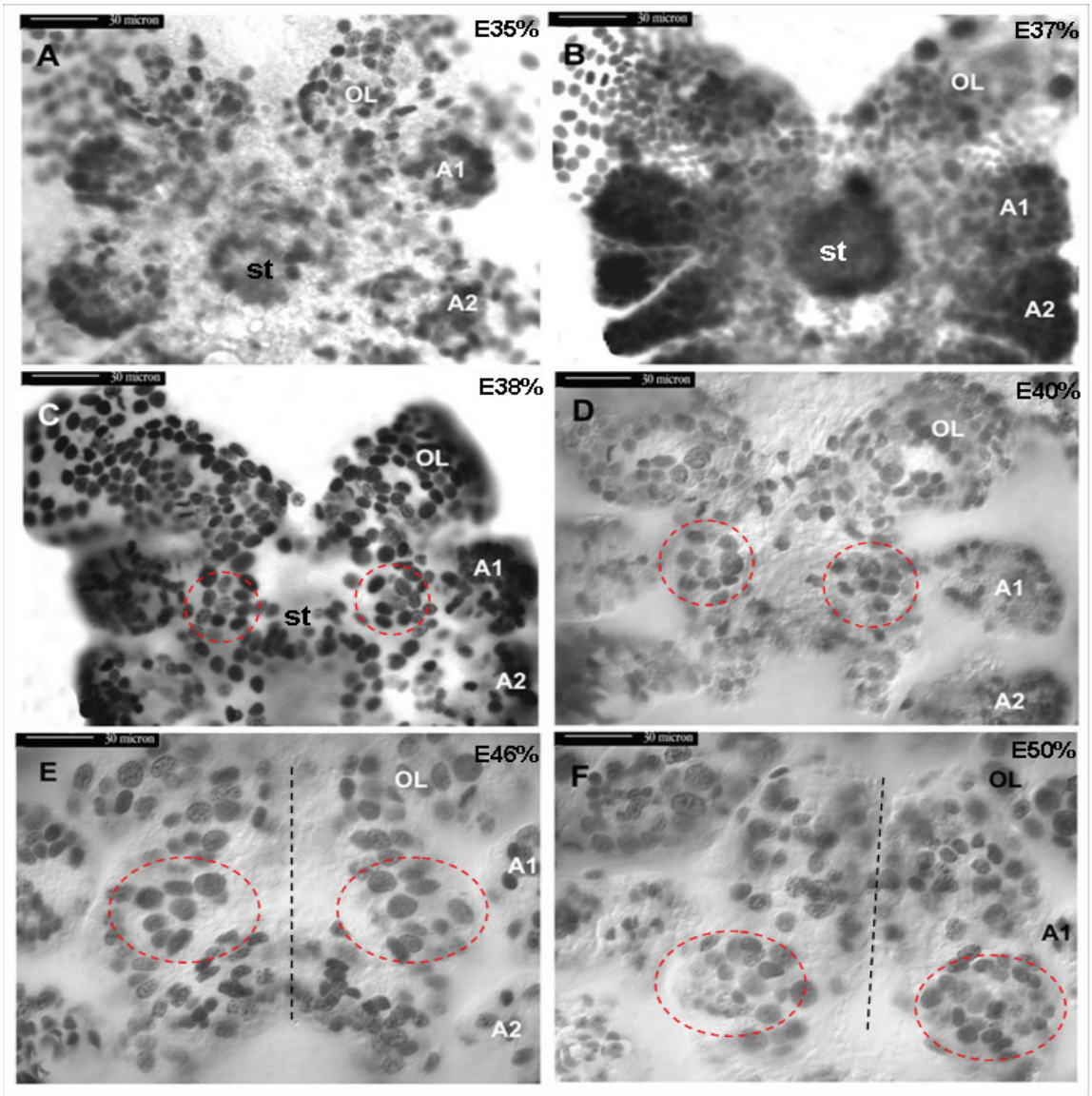


Plate 2.6- Brains at different developmental stages. BrdU staining. "Rosetta" deutocerebral region: red dotted circle. Brain midline: black dotted line. Abbr: OL, optic lobes; A1, antenna I; A2, antenna II.

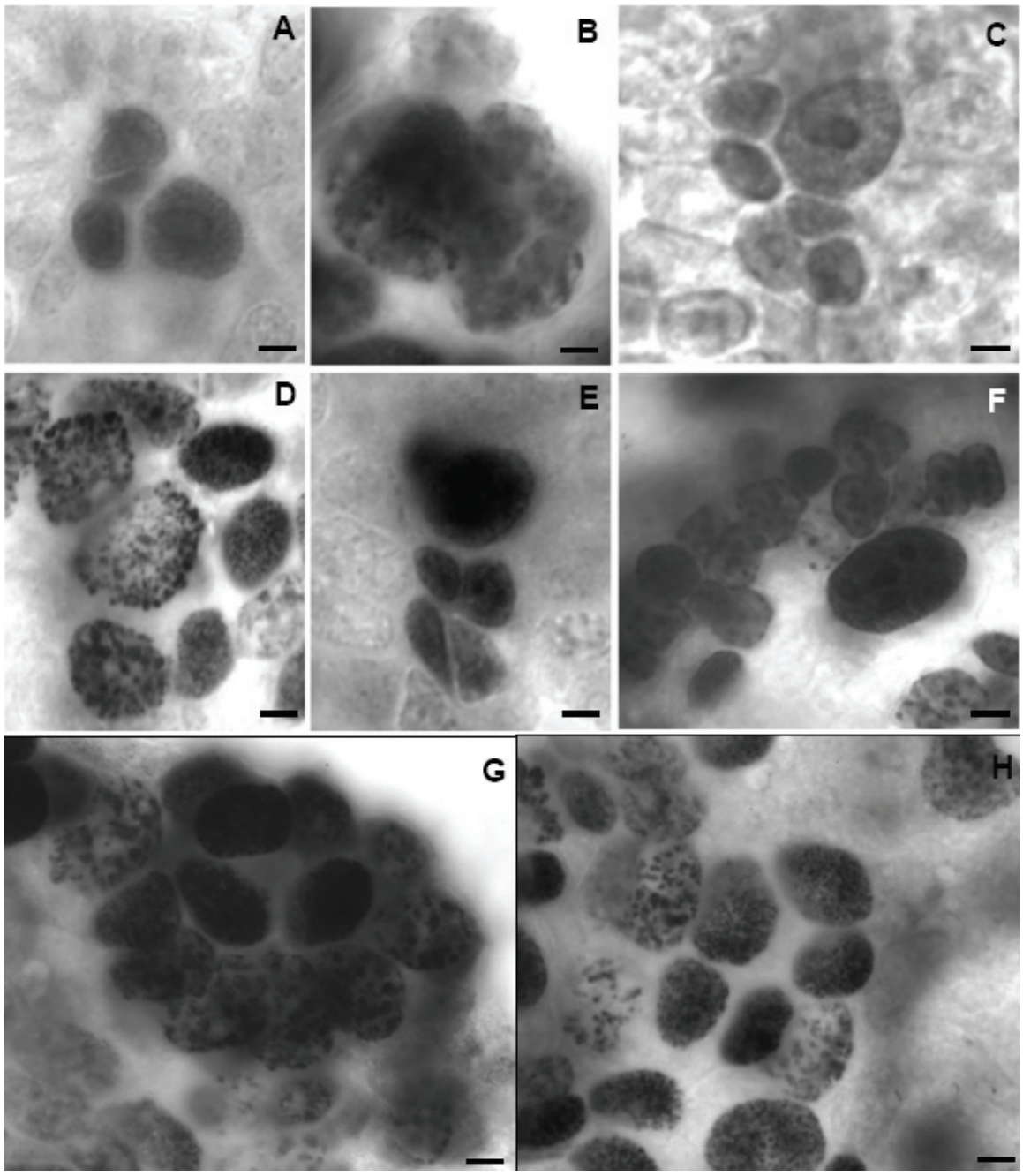


Plate 2.7- Higher magnification of neuroblasts and associated progeny. BrdU staining. Scale bar: 10μm.

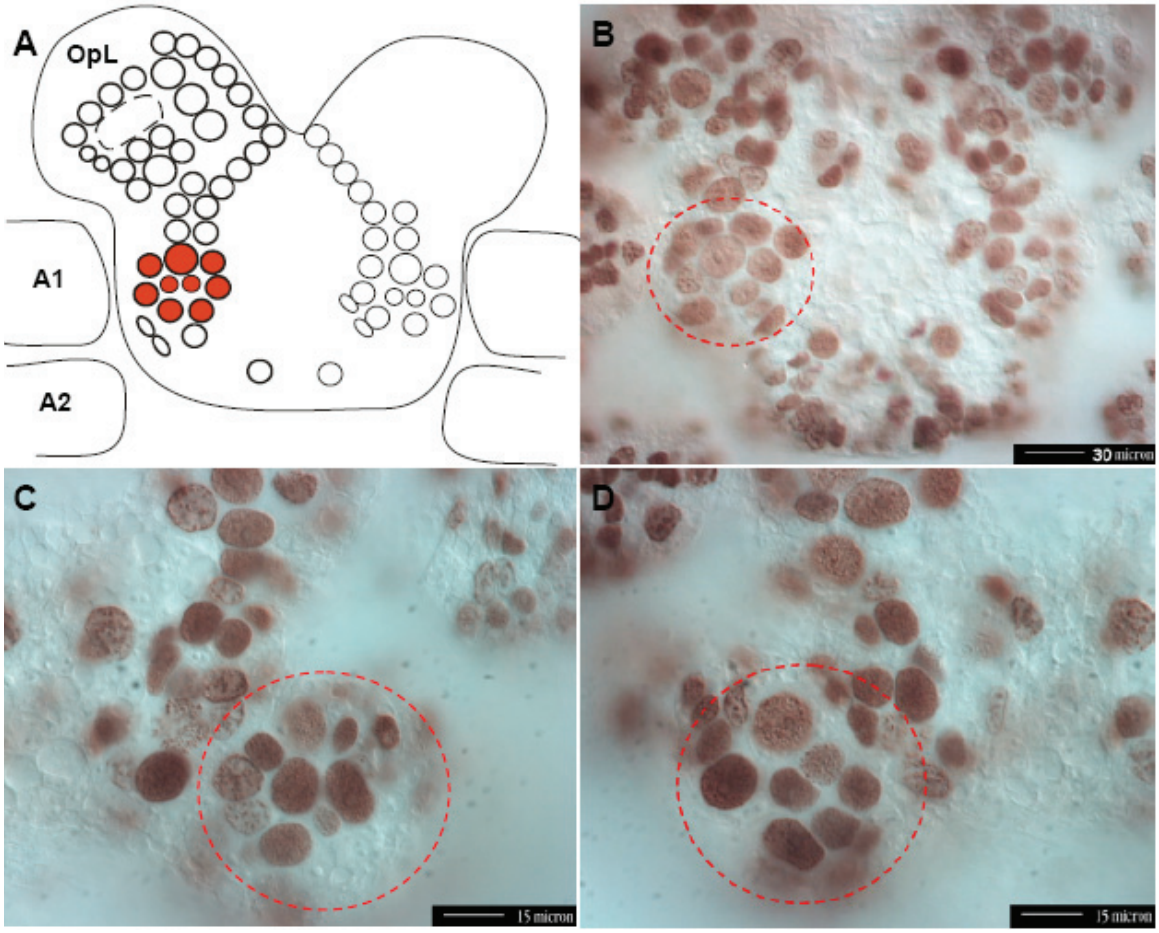


Plate 2.8- Schematic diagram adapted from a *camera lucida* drawing of the brain of the freshwater Marbled crayfish (*Procambarus* sp.) embryos at about E42% (A). B: overview of the brain at the same developmental stage of the diagram. C-D: higher magnifications of the rosetta. Abbr: OpL, optic lobes; A1, antenna I; A2, antenna II. Rosetta: red cells and red dotted circle.

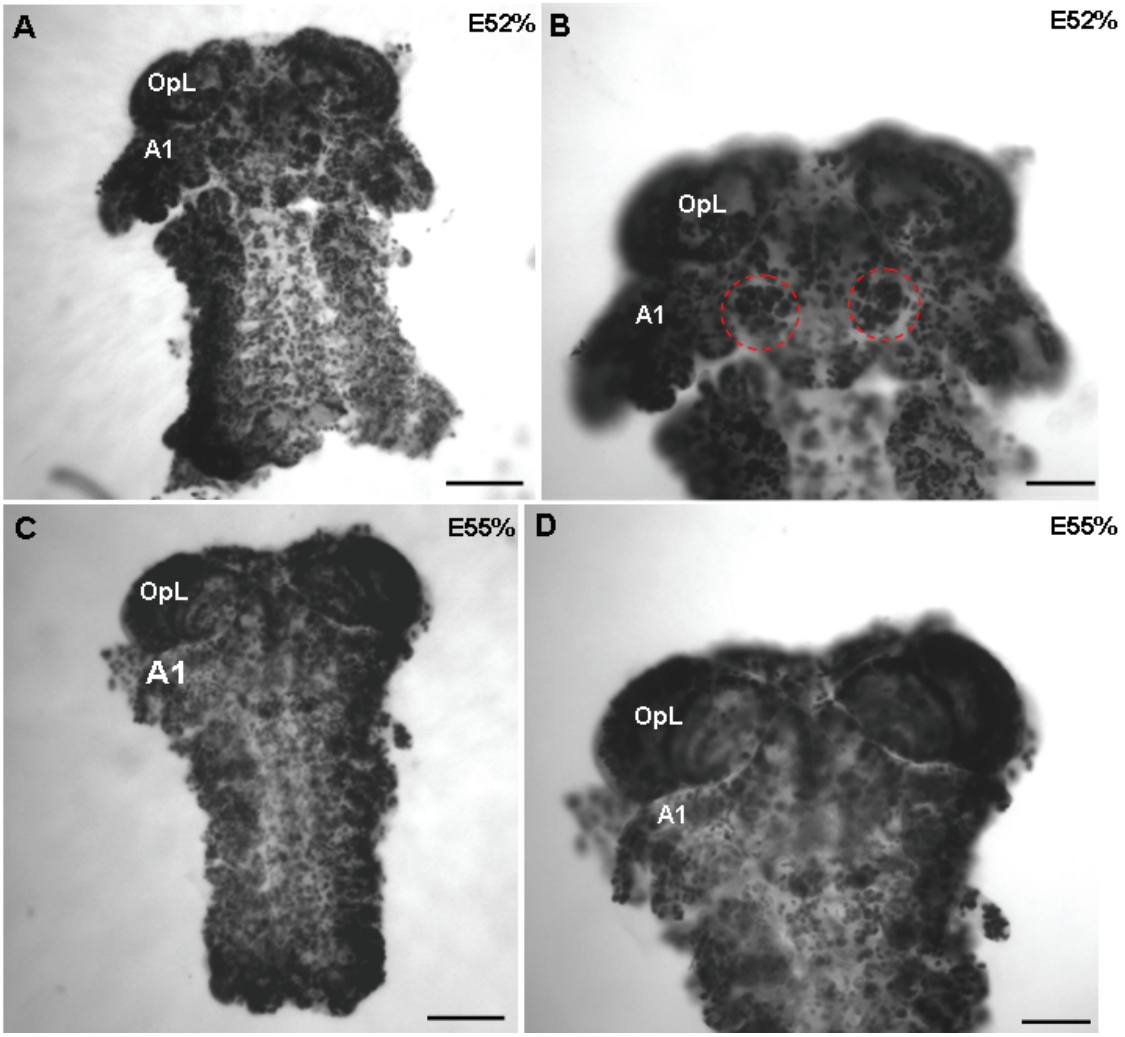


Plate 2.9- Embryos of the freshwater Marbled crayfish (*Procambarus* sp.). BrdU staining. A-B: E52%. C-D: E55%. Abbr: OpL, optic lobes; A1, antenna I. Scale bar 60µm.

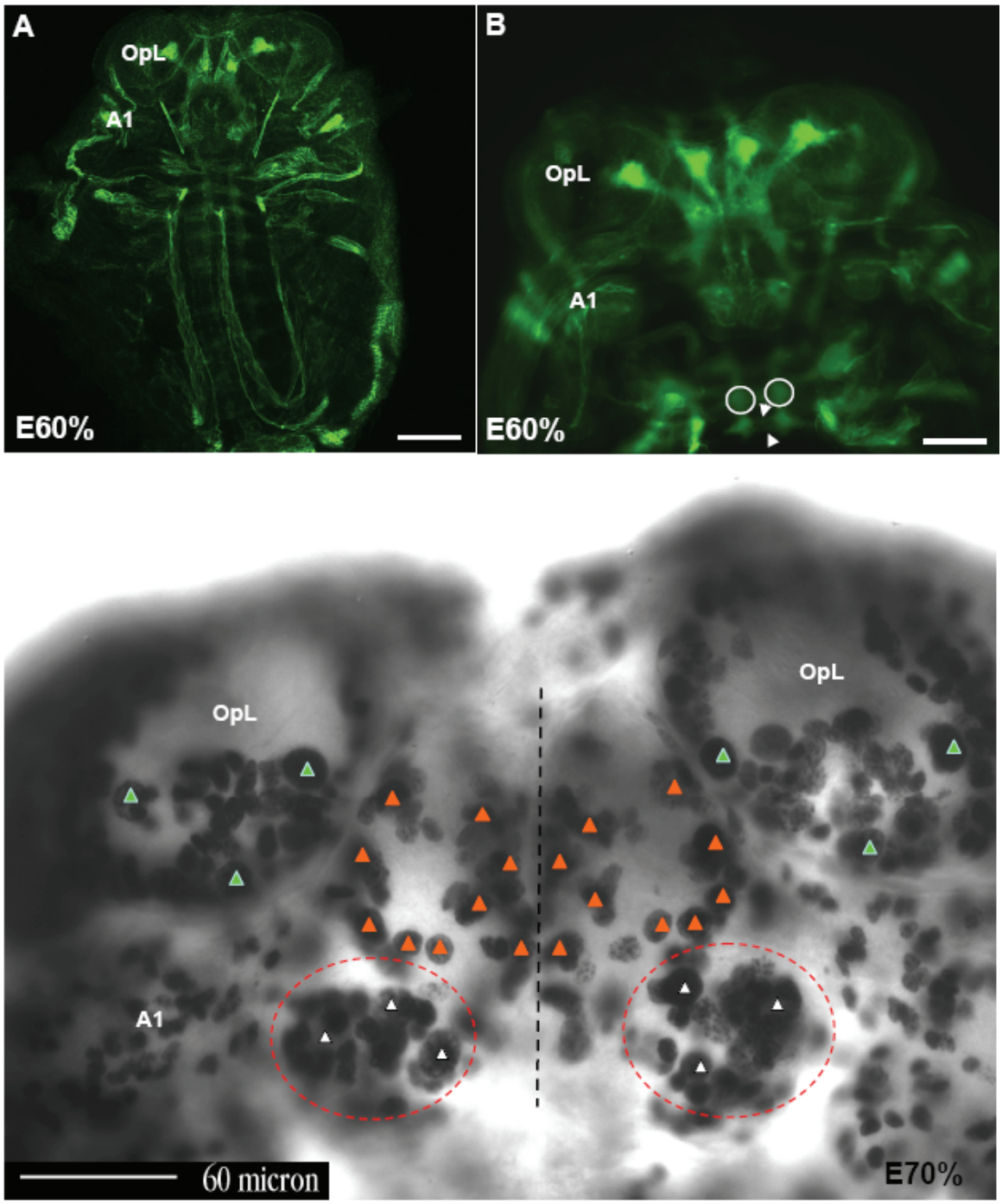


Plate 2.10- Embryo of the freshwater Marbled crayfish (*Procambarus* sp.). A-B: E60%, phalloidin staining. Ganglia anlagen: white circle. Anterior and posterior commisures: white arrowhead. C: E70%, BrdU staining. Deutocerebral proliferative system: red dotted circle. Symmetrical cells in the optical lobes (green triangle), proto- (orange triangle) and deuto-cerebrum (white triangle) in respect to the midline (black dotted line). Abbr: OpL, optic lobes; A1, antenna I; A2, antenna II; MD, mandible; MX1, maxilla I; MX2, maxilla II; cp, caudal papilla. Scale bar: 60µm.

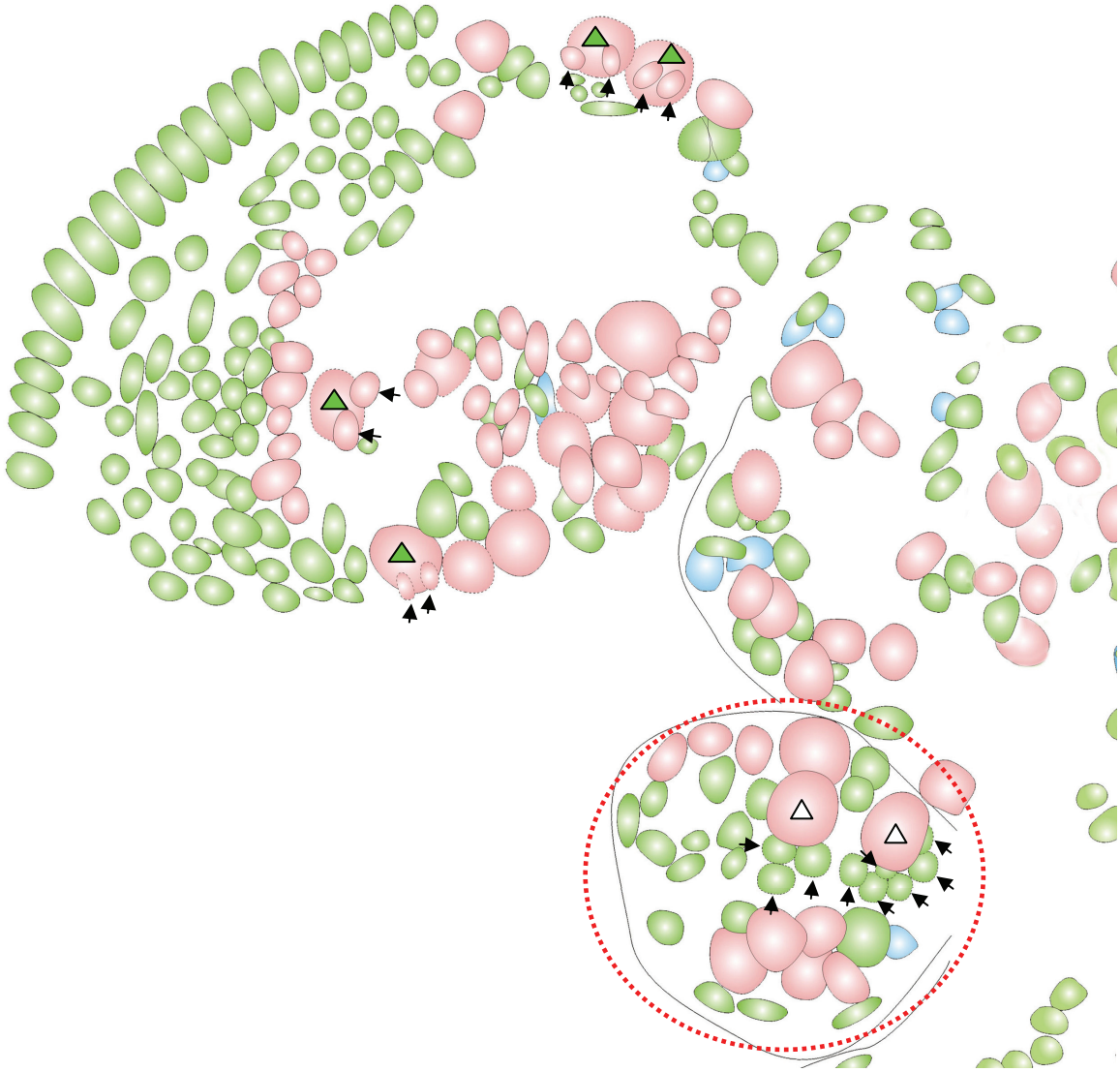


Plate 2.11- Schematic diagram adapted from a *camera lucida* drawing of the Marbled crayfish brain of Fig. 9C. The left side of the brain is represented. Deutocerecrum NBs: white triangle. Optil lobe NBs: green triangle. Black arrow: NBs progeny. Red dotted line: deutocerebral proliferative system. Color coding: ventral cells are in pink, dorsal cells are in blue, in between are cells in green.

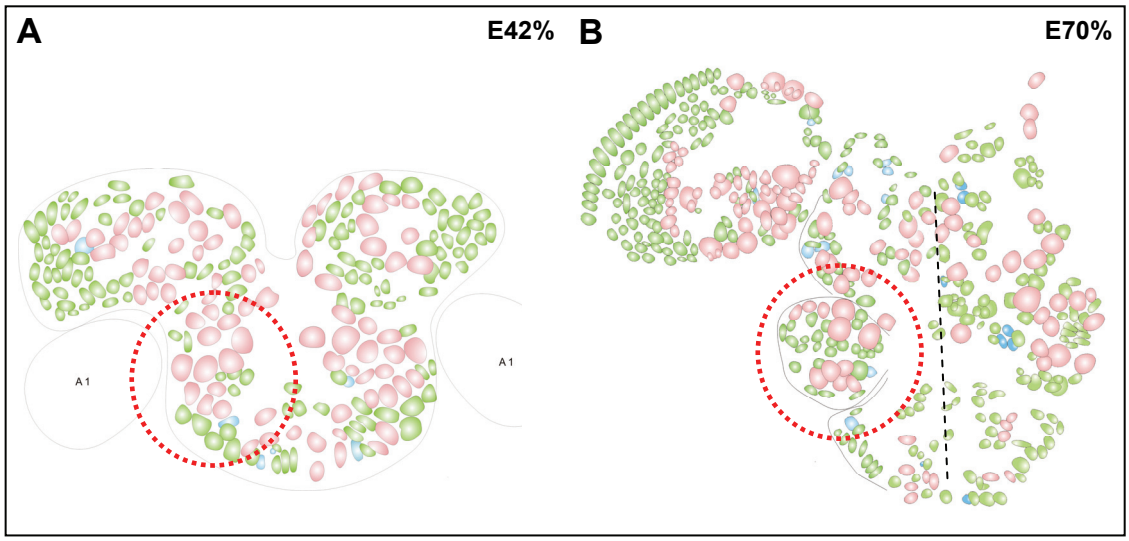


Plate 2.12- Schematic diagram adapted from a *camera lucida* drawing of embryos of the freshwater Marbled crayfish (*Procambarus* sp.). Drawings of the brain at E42% (A) and E70% (B). Comparison of the brain structure and arrangement of cells during development. Color coding: ventral cells are in pink, dorsal cells are in blue, in between are cells in green. Midline: black dotted line. Rosetta (deutocerebral proliferative system): red dotted line). A1: antenna 1.

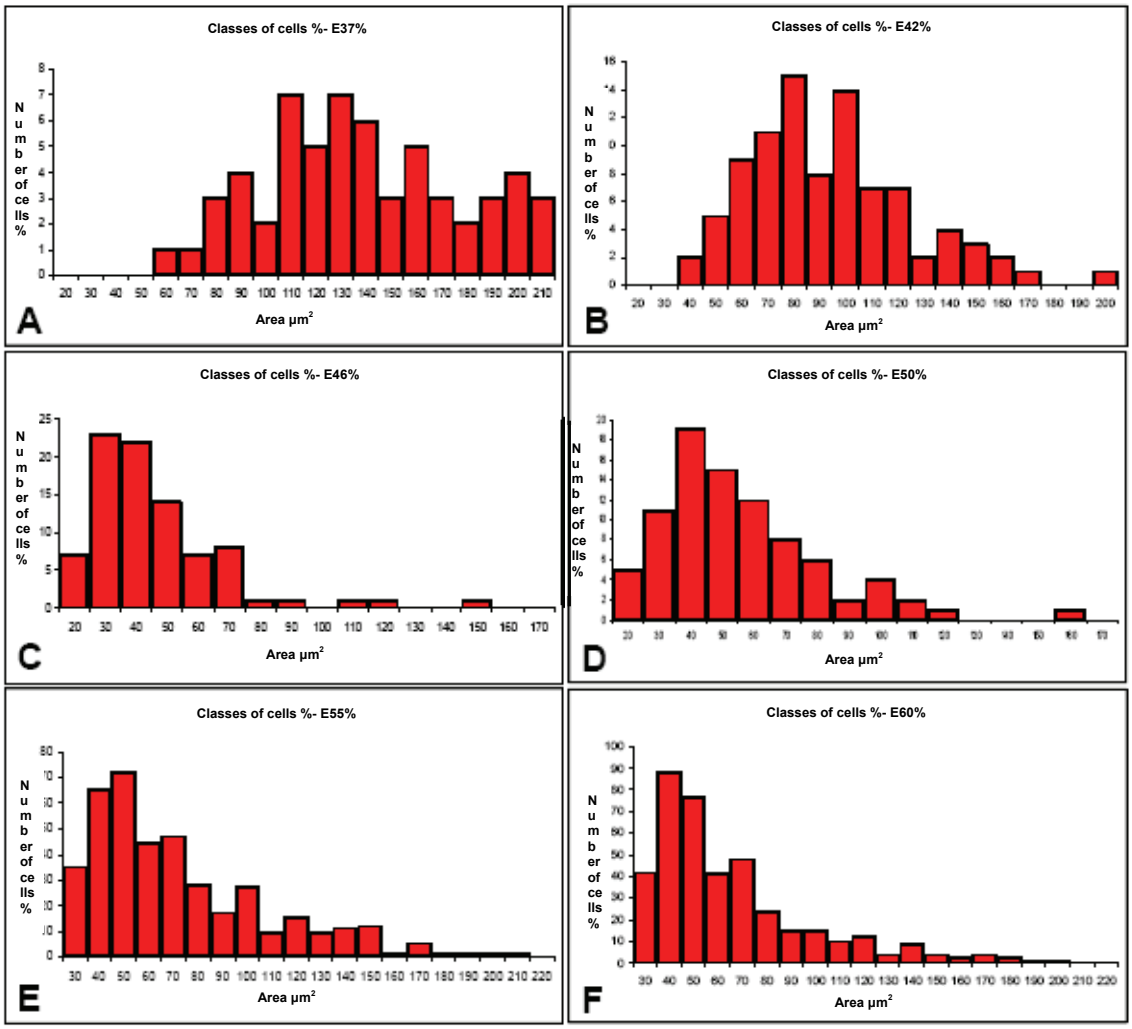


Plate 2.13- Statistical analysis based on size-classes of cells of specimens from early to late development.

2.3 DISCUSSION

Most of research on embryonic development of Crustacean nervous system has focused on more easily identifiable cells in the ventral nerve cord. In contrast, the brain, an extremely complex structure, does not vaunt such amount of studies. Little is known about brain neuroblasts in Crustacea, as well as their progeny and differentiation of single neurons: neither the number of brain neuroblasts, nor their arrangements in the three major parts of the embryonic brain, nor the cell biological mechanisms by which these neuroblasts generate their progeny are known. Mostly anatomical observations suggested that neurons of the embryonic brain may be generated by neuronal stem cell, called neuroblasts, in a mode similar to that observed in the ventral nerve cord or in Insecta. In the ventral nerve cord of crustacean, in fact, the stem cell mode usually described for insects has also been reported from the crustacean CNS (Scholtz 1990, 1992, Harzsch and Dawirs 1994, 1996a, Whitington 1996, Dohle and Scholtz 1997; Harzsch et al. 1998, Geberding and Scholtz 2001, Harzsch 2001). Regarding the brain, the scenario is not clear yet. According to Gerberding and Scholtz (2001), for instance, not all post naupliar material is produced by ectoteloblasts in Crustacea. Maxilla 1 and 2 and the first thoracic segment are formed by no-teloblastic origin cells. Nevertheless, these cells behave like ectoteloblast progeny because they go through a stereotypic pattern of differential cleavage and eventually form NBs (Gerberding and Scholtz, 2001). Thus, homologous NBs (as well as homologous neurons, i.e. *engrailed*) are generated by different cellular lineages. In fact, a distinct cell lineage is not a compulsory requirement for NBs that can derive from different types of cells. Where the brain NBs originate is a crucial point that need to be understood. In the egg-nauplius stage, the caudal growth zone is responsible for the addition of new segments to the embryo, due to the proliferative action of ectoteloblasts which give rise to NBs as well. In the brain of the Marbled crayfish such succession of different generations of cells has not been detected. Looking back at the very early stages (Pl. 2.1, 2.2), the brain appears to shape from the aggregation of neuroectodermal cells that somehow aggregate and outline the embryo but without forming any stereotype pattern like the grid-like cell arrangement seen in the ventral nerve cord. Whether these first cells are neuroblasts or ectoteloblasts or another cell type

is, therefore, not known. Scholtz (1992) showed that the egg-nauplius derives from a V-shaped germinal disc just above the caudal papilla which appears contemporaneously. The identity of cells is not discussed and ectoteloblasts are mentioned to be present only in the caudal papilla. The emergence of neuroblasts in the ventral nerve cord is thus closely related to the presence of ectoteloblasts which are identified as such because they are larger than their neighbors, they have larger nuclei and they are characterized by an unequal mode of division in an exclusively anterior direction (Scholtz, 1992). These types of data did not come out from the current study or from the literature of the egg-nauplius brain in Crustacea, so it appears that anterior head segments are not generated by ectoteloblasts. The conclusion that can be draw from these preliminary results is that NBs are likely to be present and to participate to neurogenesis but where they come from at first is not clear yet. Certainly, more investigations are needed to understand which is the identity of first cells that give the start to the brain formation, it is in fact possible that the fate of a certain cell to become neuroblast is not due to specific pattern division (as seen in the ventral nerve cord of Crustacea), but, as in insects, neuroblasts acquire their identities by spatial and temporal cues in the neuroectoderm. This may represents another interesting point of similarity between crustaceans and insects.

The main hypothesis to the current study was that three different classes of BrdU+ cells are present in the brain of the Marbled crayfish: small (neurons), medium (ganglion mother cells) and bigger cells (NBs). The statistical analysis based on size-classes of cells of all analyzed specimens from early to late development did not resolve the matter (Pl. 2.13). A marked separation of cell classes was expected to be the outcome of the analysis, instead of the contiguous imaginary line resembling the Gaussian curve that showed up from the histograms. The scenario that was drawn from the area measurement analysis is that of a mixed group of cells with different areas, ranging from small to large but with no clear distinction in classes, although a kind of separation was arbitrarily done: small (20-50μm^2), medium (50-150μm^2), large (larger than 150μm^2). In the same species, Fabritius-Vilpoux and colleagues (2008) counted, measured and mapped from 38 to 65% of embryonic development Engrailed positive cells in the neuroectoderm of the ventral nerve cord. Authors identified cells of variable sizes as well: large, intermediate, small, between large and intermediate and between intermediate and small. Besides, many

Engrailed positive putative neuroblasts and putative neurons were identified because not only size criteria were used but positional data as well. In the current study, most of the large cells are placed ventrally in the neuroectoderm layer comparing to medium and smaller cells which are located dorsally, deeper within the embryo (Pl. 2.11, 2.12). It is known that, contrary to insects, in crustaceans NBs remain in the outer cell surface. This is also visible by the stable presence, on the ventral side, of some identifiable NBs (i.e. rosetta) throughout the entire embryogenesis process. Therefore, it may be suggested that these groups of ventral large cells are neuroblasts. Moreover, as seen in Pl. 2.5B and Pl. 2.10A, brains stained by phalloidin reveal the presence of axonal tracts, which means that neurons are present since early stages of embryogenesis, and these must have been generated by precursor cells. According to what has been seen in the ventral nerve cord, it can be proposed that neuroblasts are the precursor cells. Scholtz (1992), by analyzing transversal sections of the mandibular, maxillary and thoracic ganglionic segment of *Cherax destructor* embryos, described columns of ganglion mother cells from neuroblasts. The presence of columns of daughter cells has not been detected in the current study. In the deutocerebrum for instance, large, spherical cells have been found tightly surrounded by smaller cells that appeared to lie all in the same focal plane (but slightly more dorsally compared to the putative NB), and not deeper into the tissue as a columnar structure would infer.

In lobster embryo (Benton and Beltz, 2002), the use of the BrdU technique showed the existence of three distinct cell types of cells involved in neurogenesis but, unlike the insects model in which ganglion mother cells divide once, intermediate precursor of embryonic lobster divide multiple time to give rise to neurons. These authors found BrdU+ precursors with “strings of smaller progeny trailing away from the large BrdU cells” in the thoracic ganglia. By contrast, in the brain, BrdU+ precursors were found to be surrounded by cluster of cells. This is in accordance to the scenario seen in the Marbled crayfish brain in which no column but cluster of cells are associated in a circular manner to the putative NB, suggesting a different mode of migration of daughter cells within the tissue. In fact, it needs to be recalled that crustacean neuroblasts, after generating ganglion mother cells, can produce also epidermal cells because they are not committed to neuronal fate (Dohle and Scholtz, 1988). This may explain why a

stereotype pattern of cell columns towards the inside of the embryo is not visible. Some cells may remain in the outer surface as epidermal cells.

In the Marbled crayfish brain of older embryos, neuroblasts are accommodated in two different modes: isolated and patchily distributed in the protocerebrum and tritocerebrum and grouped together in the deutocerebrum and optic lobes where set of NBs are arranged in circle (rosetta) or semicircle. Actually this is not meant to be a strict classification of NBs based on their arrangement but simply a description of how NBs appear as development reaches the later stages. The rosetta cluster of putative NBs in fact is a constant feature of the deutocerebrum during embryogenesis. In grasshopper, during morphogenesis, not single but groups of neuroblasts move together, giving origin to the main subdivisions of the developing brain (Boyan et al., 1995). It is reasonable to assume that at least the deutocerebral rosetta is made of a cluster of neuroblasts that since the beginning of neurogenesis forms the main structure of the deutocerebrum and delineates this part from the proto- and tritocerebrum. In the proto- and tritocerebrum, no specific local pattern was noticed during embryogenesis but left-right symmetry was clearly displayed by single labeled cells along the midline and together with single dispersed putative NBs in the main brain compartments of the Marbled crayfish. An attempt of map of the main larger and medium cells was done but more detailed information need to be provided in order to reach the same level of precision obtained for insects. In fact, Zacharias and colleagues (1993) observed, during early neurogenesis of *Drosophila*, approximately 130 mitotically active neuroblasts in predictable positions in each brain hemisphere. For these reason, a map of single identifiable neuroblasts was created and now is available for insects (Zacharias et al., 1993). Neuroblasts have been not only counted but each of them has its own “identity card” which reports reactivity to specific molecular marker, antibodies and positional information is reported as well. Similar studies have been carried out in grasshopper by Boyan and collegues (1995) who identified individual neurons that pioneer the initial projections in the embryonic brain and determined their lineage from identified brain neuroblasts. These studies indicated that most likely the complexity of the mature grasshopper brain derives from an extremely simple and stereotyped set of neuronal precursor cells. Same conclusions apply for *Drosophila* and different insect species whose basic aspects of the spatial and

temporal development of the embryonic brain neuroblast pattern are conserved (Urbach and Technau, 2003). Actually, whether NBs in crustaceans are homologous to those in insects is still an unsettled question (Dohle and Scholtz 1988, Scholtz 1992, Whitington 1996, Dohle 2001, Scholtz and Gerberding 2002, Harzsch 2003c, 2006, Duman-Scheel and Patel 1999, Richter 2002). The debate is based on their different mode of generation and the fact that cell lineages of identified neurons from individual NBs have not been defined yet. Only recently, in the ventral nerve cord of the crustacean *Orchestia cavimana*, a partial neuroblasts map was established based on the identification of lineages from individual NBs to identified pioneer neurons, suggesting homology of NBs and their lineages to that of *Drosophila* (Ungerer and Scholtz, 2008*)*. In conclusion, studies on insects (i.e. the fruit fly *Drosophila melanogaster*, the grasshopper *Schistocerca gregaria*, the moth *Manduca sexta*, the beetle *Tribolium castaneum*) and malacostracan crustaceans showed that neuroblasts drive neurogenesis, although with different modes of division, according to the species or the compartment of the brain. In lobster (Benton and Beltz, 2002) and in the spider crab (Harzsch and Dawirs, 1995a) embryos, for instance, the three different classes of cells have been clearly demonstrated to be present and follow the stem cell mode, though with different dynamics compare to that in insects. In the Marbled crayfish, larger and smaller cells have been found to be sometime associated to each other suggesting a relationship NB-daughter cell, but the majority of "medium size" cells has not been clarified in term of identity (are they ganglion mother cells or bigger neurons? Are they small or medium NBs?).

2.4 CONCLUSIONS

This study was conceived as preliminary project to get into the embryogenesis of the Marbled crayfish, to get to know the main structures of the brain, the concepts of neuroblasts, cellular divisions, arthropods phylogeny and, even more, to practice the immunohistochemical techniques which allow the investigation of neurogenesis. Most of the questions arose in the discussion need further experiments, diverse techniques and approaches. Identification of ectoteloblast, NBs and progeny requires the use of molecular markers or specific antibodies. Serial sections may help in discovering whether

chains of ganglion mother cells are effectively produced by superficial NBs within the embryo. The use of a double staining with specific neurons antibodies or simply by phalloidin may add more information as well as comparisons with other arthropods groups.

Chapter 3

Engrailed

3.1 INTRODUCTION

Hexapoda have been traditionally seen as the closest relatives of the Myriapoda (Tracheata hypothesis) but molecular studies have challenged this hypothesis and rather have suggested a close relationship of hexapods and crustaceans (Tetraconata hypothesis). In this new debate, data on the structure and development of the arthropod nervous system contribute important new data ("neurophylogeny"). Neurophylogenetic studies have already provided several examples for individually identifiably neurons in the ventral nerve cord that are homologous between insects and crustaceans. In the present report, we have analyzed the emergence of Engrailed-expressing cells in the embryonic brain of a parthenogenetic crayfish, the Marbled crayfish (Marmorkrebs), and have compared our findings to the pattern previously reported from insects. Our data suggest that a group of six Engrailed-expressing neurons in the optic anlagen, the so-called secondary head spot cells can be homologized between crayfish and the grasshopper. In the grasshopper, these cells are supposed to be involved in establishing the primary axon scaffold of the brain. Our data provide the first example for a cluster of brain neurons that can be homologized between insects and crustaceans and show that even at the level of certain cell groups, brain structures are evolutionary conserved in these two groups.

3.1.1. The Engrailed-expressing secondary head spots in the embryonic crayfish brain: examples for a group of homologous neurons in Crustacea and Hexapoda?

Several examples are available of individually identified neurons in the ventral nerve cord that were suggested to be homologous between decapod crustaceans and hexapods (for review, see Harzsch 2003a, Harzsch et al. 2005). Despite of these examples from the ventral nerve cord, so far, we do not have any descriptions of identified neurons in the brain that may be homologous between Crustacea and Hexapoda. The segment polarity gene *engrailed* (*en*) encodes a homeobox transcription factor and during segmentation of Arthropoda is expressed by cells in the posterior part of a segment and later on during development also in the nervous system (Patel et al., 1989). In crustaceans, the embryonic expression of *en* during segmentation of various malacostracan taxa has been

analyzed (Patel et al. 1989, Scholtz et al. 1993, 1994, Scholtz 1995b, Abzhanov and Kaufman 2000, Harzsch 2003b, Alwes and Scholtz 2006, for review, see Patel 1994, Scholtz, 2001). In crayfish, the localization of Engrailed (En) proteins during germ band formation has been studied in *Procambarus clarkii* (Patel et al., 1989) using the monoclonal antibody 4D9 and has also been briefly documented in the Marbled crayfish (Alwes and Scholtz, 2006). A first single-cell analysis of neuronal *en* expression comparing Malacostraca and Insecta has been undertaken by Duman-Scheel and Patel (1999) who showed several individually identified En-positive (En+) neurons to be homologous throughout the two groups suggesting that this approach is well suited for phylogenetic inferences. Therefore, we focused on En expression in the developing crustacean brain in the present report. Neuronal En expression has already been thoroughly analyzed in embryonic insect brains (e.g. Schmidt-Ott and Technau 1992, Schmidt-Ott et al. 1994, Boyan and Williams 2002, Urbach and Technau 2003, Urbach et al. 2003) so that these studies may serve as a good basis for comparisons with crustaceans. In search of brain neurons that may be homologous between insects and crustaceans, we analyzed the emergence of a specific group of protocerebral cells that express the En protein, the secondary head spots, by immunohistochemistry in embryos of the parthenogenetic Marbled crayfish (Marmorkrebs; Malacostraca, Decapoda, Astacida), an unidentified crayfish of uncertain geographical origin the closest relatives of which seem to belong to the genus *Procambarus* (Scholtz et al., 2003). This is an outstanding crustacean because it provides the first example for parthenogenesis within the decapod crustaceans (Scholtz et al. 2003, Seitz et al. 2005, Martin et al. 2007). The Marbled crayfish reproduces by apomictic parthenogenesis, the most common asexual reproductive mode among invertebrates (Martin et al. 2007). By apomictic propagation, the offsprings conserve the original genetic information and, thus, are identical clones of the mother (Martin et al. 2007). Due to its high fertility and mode of reproduction, it is an excellent model to explore aspects of crustacean embryology (Seitz et al. 2005, Alwes and Scholtz 2006, Vilpoux et al. 2006, Rieger and Harzsch 2007). Our results suggest that a certain group of En+ neurons in the insect protocerebrum (PC) has homologous counterparts in the crayfish brain.

3.2 RESULTS AND DISCUSSION

3.2.1. Spatiotemporal pattern of Engrailed expression in the early embryonic brain

In the early embryonic brain of the Marbled crayfish, the Engrailed expression starts at around 35–37% of embryogenesis (E35–E37%) and can be detected until E55% (staging system according to Seitz et al., 2005; Pl. 3.1 and 3.2a–i). In the ventral nerve cord, there are segmentally iterated stripes of En+ cells that span across the width of the segment and that are located at the posterior neuromere borders as exemplified in Pl. 3.1b for the mandible (MD), maxilla 1 (MX1) and maxilla 2 (MX2) segments. The arrangement of En+ cells during segmentation of the germ band and neurogenesis in the ventral nerve cord has been thoroughly documented for the crayfish and will not be touched in this study (Patel et al. 1989, Alwes and Scholtz 2006). As in the ventral nerve cord, segmentally iterated groups of En+ cells are present in the brain, presumably arranged close to the posterior borders of the neuromeres with one cluster associated with the PC, one with the deutocerebrum (DC) and a third group with the shape of a stripe associated with the tritocerebrum (TC), on both sides of the brain (Pl. 3.1b, 3.2). We will call these the primary protocerebral (ppc), primary deutocerebral (pdc) and primary tritocerebral (ptc) groups in the following. This arrangement shows distinct similarities to the labeling pattern described by Scholtz (1995b) for *Cherax destructor*. Compared to the mandible, maxilla 1 and maxilla 2 in which the En+ cells are arranged in transverse stripes, in the Marbled crayfish brain, the labeled cells are situated in bilateral symmetrically arranged clusters. From E37% to E39%, the protocerebral and deutocerebral clusters are composed of between five and six En+ cells and the paired tritocerebral stripe is composed of approximately eight to ten cells per hemibrain (Pl. 3.2a,b,j,k). All three clusters of cells do not extend across the midline of the brain where the stomodaeum starts to develop. This general pattern of the primary clusters will remain the same for a further 12–15% of embryonic development (Pl. 3.2).

3.2.2. Engrailed expression in the advanced embryonic brain (E41–E45%)

In the primary protocerebral and deutocerebral groups, the number of small cells slightly increases as development proceeds (Pl. 3.2d–f). In addition, at E44%, one single cell

appears in each hemisphere laterally in the PC (Pl. 3.2g, 3d). The cells in the primary clusters appear to be of roughly similar size (30 to 50µm2) with the exception of one or two conspicuous, much larger cells per cluster (80 to 100µm2; Pl. 3.2k–m). From the primary clusters, newly emerging cells of an elongate shape seem to extend laterally along the posterior borders of the optic anlagen and antenna 1 (Pl. 3.2d to f, 3b–d and 4a). By E41%, the primary protocerebral cluster is associated with a single row of En+ cells that encircles the optic anlagen (Pl. 3.2d,e,j). By E43%, this row has enlarged to two or three cells (Pl. 3.2f, 3.4a). In parallel, the number of En+ cells at the posterior side of the deutocerebral appendage, the antenna 1, also increases (Pl. 3.2e,f). In the tritocerebral neuromere, on both sides, the En+ cells are arranged in a double row, which laterally extends towards the associated antenna 2 (Pl. 3.2f,j). These stripes medially are not connected because of the stomodaeum.

3.2.3. Emergence of secondary head spots and tertiary head cells: comparison to other Crustacea

Between E40% and E50%, a new set of En+ cells gradually appears in the optic anlagen in addition to the primary protocerebral groups (Pl. 3.3, 3.4). In accordance with Schmidt-Ott and Technau (1992) who observed these cells in an insect and with Scholtz (1995a, b) who studied crayfish, we will call them the **secondary head spot cells** (shs). At E40%, on both sides, a first single cell emerges in the middle of the optic anlagen (Pl. 3.2c, 3a). Between E42% and E45%, three more cells appear close to the first cell and form a column (Pl. 3.2e–h, 3.3b–e). In some specimens, this column of cells extended posteriorly towards the border of the optic lobes. Cells on the tip of the column are most strongly labeled (Pl.3.3c,d). By E50%, a sixth cell has appeared in the cluster of secondary head spot cells (Pl. 3.3f). From E45% onwards, the strength of labeling in the primary protocerebral and deutocerebral clusters diminishes (Pl. 3.2i). Double labeling of actin and En revealed that the secondary head spots are closely associated with the developing neuropil in the optic anlagen. They are located slightly posterior to the joint anlagen of the medulla terminalis and hemiellipsoid body (Pl. 3.4; see Vilpoux et al., 2006 for the structure and nomenclature of the embryonic optic neuropils). Starting at E45%, additional En+ cells appear in the optic anlagen that we will term the tertiary head

cells (Pl. 3.2h, 3.4d). The first tertiary head cell appears slightly anterior to the hemiellipsoid body anlagen, which is tightly associated with it (Pl. 3.2h, 3.4a). Higher magnifications show that this cell seems to be embedded superficially in the developing neuropil (Pl. 3.4e). The second and third cells emerge more posteriorly and closer to the secondary head spot cluster. At E48%, all three tertiary head cells have appeared and are frequently arranged in an arc (Pl. 3.4d). Whereas in the previous studies by Patel and colleagues (1989) and by Abzhanov and Kaufman (2000) on Engrailed expression in crayfish, the secondary head spot cells were not taken into account, Scholtz (1995b) briefly mentions these cells in *Cherax destructor* embryos and identified them as epidermal cells. Our results in the Marbled crayfish brain show distinct similarities to the labeling pattern described by Scholtz (1995b) for *Cherax destructor* in that, in both species, clusters of En+ cells in the brain (the "primary" groups) are located at the posterior borders of the neuromeres with one cluster being associated with the TC, one with the DC and one with the PC, on both sides of the brain. Contrary to Scholtz (1995b) who suggested that the Engrailed patterns of the first and second antennae resemble each other closely, we rather found slightly more similarities between the protocerebral and deutocerebral primary clusters, whereas the En+ cells associated with the TC look more like a transverse row, a pattern that is reminiscent of that in the more posterior segments from the mandibular neuromere onwards.

3.2.4. The secondary head spot cells in the optic anlagen of insects

The En+ secondary head spot cells associated with the developing optic lobes have been first described in the fruit fly *Drosophila melanogaster* (Schmidt-Ott and Technau, 1992). During early embryogenesis, these authors observed a protocerebral cluster of En+ cells that they termed the "en head spot" and that resembles a stripe crossing "each brain hemisphere transversely at its ventral side". In later stages (stages 13/14), a group of cells detached from the head spot, the bilaterally arranged "secondary head spot". The secondary head spots subsequently were also analyzed in a number of different insects (for review, see Urbach et al., 2003) such as additional Dipterans and the beetle *Tribolium castanaeum* (Schmidt-Ott et al. 1994, Brown et al. 1994, Rogers and Kaufman 1996), the grasshopper *Schistocerca gregaria* (Zacharias et al. 1993, Boyan and Williams

2002) and the beetle *Tenebrio molitor* (Urbach et al., 2003). The developmental origin of the secondary head spots is different between the various insects examined so that is unclear in the moment if they represent a homologous group of cells throughout Insecta (Urbach et al., 2003). Based on the assumption that the major functional role of the *engrailed* gene is to act during segmentation, the secondary head spots have repeatedly been interpreted as delimiting an additional metameric unit anterior to the PC (e.g. Schmidt-Ott and Technau 1992, Schmidt-Ott et al. 1994, Zacharias et al. 1993, Rogers and Kaufman 1996). This idea was recently rejected by Boyan and Williams (2002) who performed a single-cell analysis of secondary head spot emergence in grasshopper embryos. Among the earliest pioneer neurons that contribute to the primary axon scaffold in the embryonic grasshopper brain are those in the optic lobes between the developing lamina and medulla (for review, see Boyan et al., 2003). The En+ head spot cells, identified as neurons, contribute to the primary axon scaffold of the brain in that they project their axons into the optic tract towards the median brain. Hence, from analyzing their ontogeny, Boyan and Williams (2002) concluded that the secondary head spot cells in grasshopper embryos do not represent a segmental border in the brain.

3.2.5. Comparing insect and crustacean secondary head spot cells

Despite the fact that the developmental dynamics of En+ cells is slightly different in the grasshopper as opposed to other insects (Urbach and Technau, 2003), we nevertheless will compare the grasshopper (Boyan and Williams, 2002) and crayfish secondary head spot cells because, for the insects, the most detailed information is available for the grasshopper. This comparison reveals striking similarities suggesting a homology of this group of cells as we will now discuss in detail. The features that lead us to this hypothesis are: similar gene expression, similar position and numbers, corresponding developmental characteristics. A similar set of criteria has been applied to homologize En+ neurons in the arthropod ventral nerve cord (Duman-Scheel and Patel, 1999). In both grasshoppers and crayfish, the secondary head spots appear considerably later than the primary groups and then last for about 20% of development. Furthermore, the Engrailed expression starts in just a single cell in both taxa. The head spots are located anterolaterally to the primary En+ protocerebral group and are associated with a developing lateral neuropil portion in

the PC that most likely is homologous between insects and crustaceans (lateral PC in grasshopper/combined anlagen of the medulla terminalis and hemiellipsoid body in crayfish; Vilpoux et al., 2006). In grasshopper, the secondary head spot cells are the first-born sibling progeny of three identified protocerebral neuroblasts, which express Engrailed before generating their lineages. The head spot cells re-express Engrailed and hence show a reacquisition of Engrailed expression during later differentiation (Boyan and Williams, 2002). As shown by Scholtz et al. (1993) in three Malacostraca species, cells that derive from progenitors, which have already ceased to express this gene, can express Engrailed *de novo*. Although the new expression is correlated with the formation of segment border, these authors suggest that the onset of secondary En+ expression is controlled by positional rather than lineage-specific cues (Scholtz et al., 1993). As in grasshoppers, a final set of six secondary head spots cells is present in crayfish. In grasshoppers, these six cells are the sibling progeny of three identified protocerebral neuroblasts, which may also be the case in crayfish, but this question needs further confirmation. We observed several large En+ cells within the primary protocerebral cluster of mostly small En+ cells. Similar to the grasshopper, these may be neuroblasts that generate the head spot cells, which only later during differentiation secondarily re-express Engrailed. One difference between the grasshopper and crayfish is that in the former species, during the peak period of expression, the primary protocerebral cluster comprises up to 18 En+ neuroblasts whereas there are much less putative En+ neuroblasts in the crayfish. A smaller number of protocerebral En+ neuroblasts is also characteristic for most other insects, indicating that the grasshopper may be exceptional in this respect (Urbach and Technau, 2003). The grasshopper secondary head spots comprise neurons that direct axonal processes into the developing optic tract and have been suggested to be involved in establishing the primary axon scaffold of the brain (Boyan and Williams, 2002). A recent study in fact has shown that in the fruit fly's ventral nerve cord Engrailed is directly involved in the regulation of axonal guidance cues (Joly et al., 2007). The fact that in crayfish the secondary head spot cells are located close to the origin of the protocerebral tract that links the eyestalk neuropils including the lateral PC (medulla terminalis plus hemiellipsoid body; see Vilpoux et al., 2006) to the median brain may

suggest that, like in insects, the crayfish secondary head spot cells may also take a role in establishing the primary axon scaffold of the brain.

3.3 CONCLUSIONS

In the field of morphology, evolutionary analyses of the nervous system more than before contribute important arguments to the revived debate on arthropod relationships and "neurophylogeny" is now a well-established term (for review, see Harzsch, 2006). Among other characters, the neurophylogenetic analysis of individually identified neurons in the ventral nerve cord has contributed arguments in favour of a close relationship between crustaceans and hexapods (for review, see Harzsch 2003a, Harzsch et al. 2005). The present report provides further evidence for striking similarities of the crayfish and grasshopper nervous systems and for the presence of evolutionary conserved structures down to the level of an identified, small cluster of cells in these two groups. We suggest that the cluster of En+ secondary head spot cells to be homologous between Insecta and Malacostraca, which implies that this cluster, despite differences in its ontogenetic emergence, is nevertheless homologous throughout Insecta. Our study can now serve as a basis for comparisons with other arthropod taxa to analyze if the secondary head spot cells are a synapomorphy of Malacostraca and Hexapoda or rather represent a plesiomorphic feature.

3.2.6. Plates

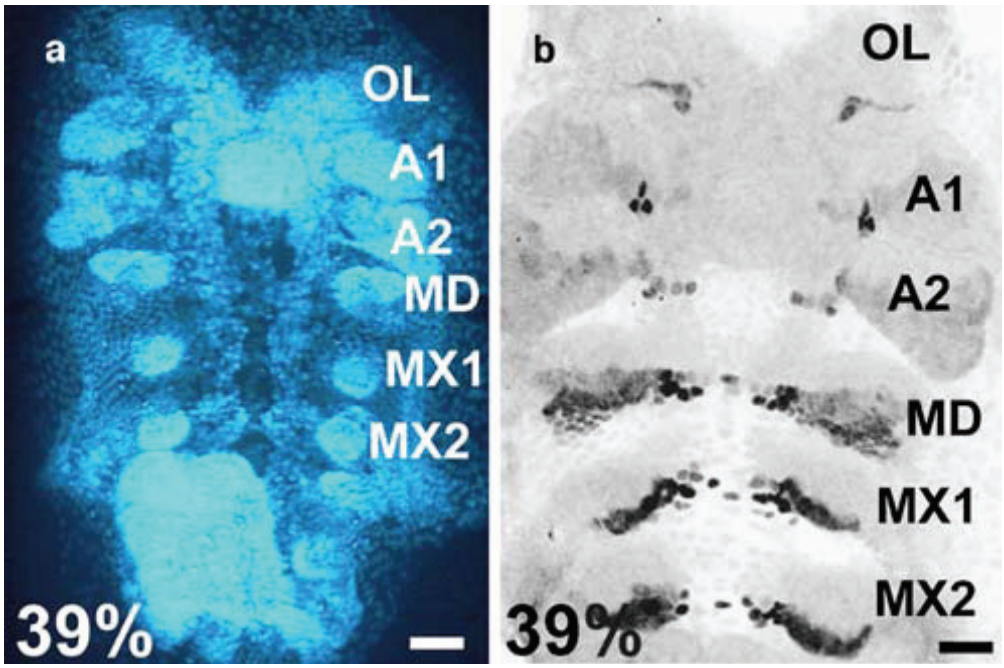


Plate 3.1- a: embryo of the marbled crayfish labelled with a fluorescent nuclear dye (HOECHST) to highlight its morphology (reprinted with permission from Seitz et al. 2005). b: Engrailed immunoreactivity in a whole mount of a E39% embryo, ventral view (anterior is to the top). In the brain, clusters of En+ cells are located at the posterior borders of the neuromeres with one cluster associated with the tritocerebrum (TC), one with the deutocerebrum (DC) and one with the protocerebrum (PC), on both sides of the brain. In the ventral nerve cord, there are segmentally iterated stripes of En+ cells located at the posterior neuromere borders. Abbr: A1 antenna one, A2 antenna two, MX1 maxilla one, MX2 maxilla two, MD mandible, OL optic anlagen. Scale bars: a 50 µm, b 25 µm.

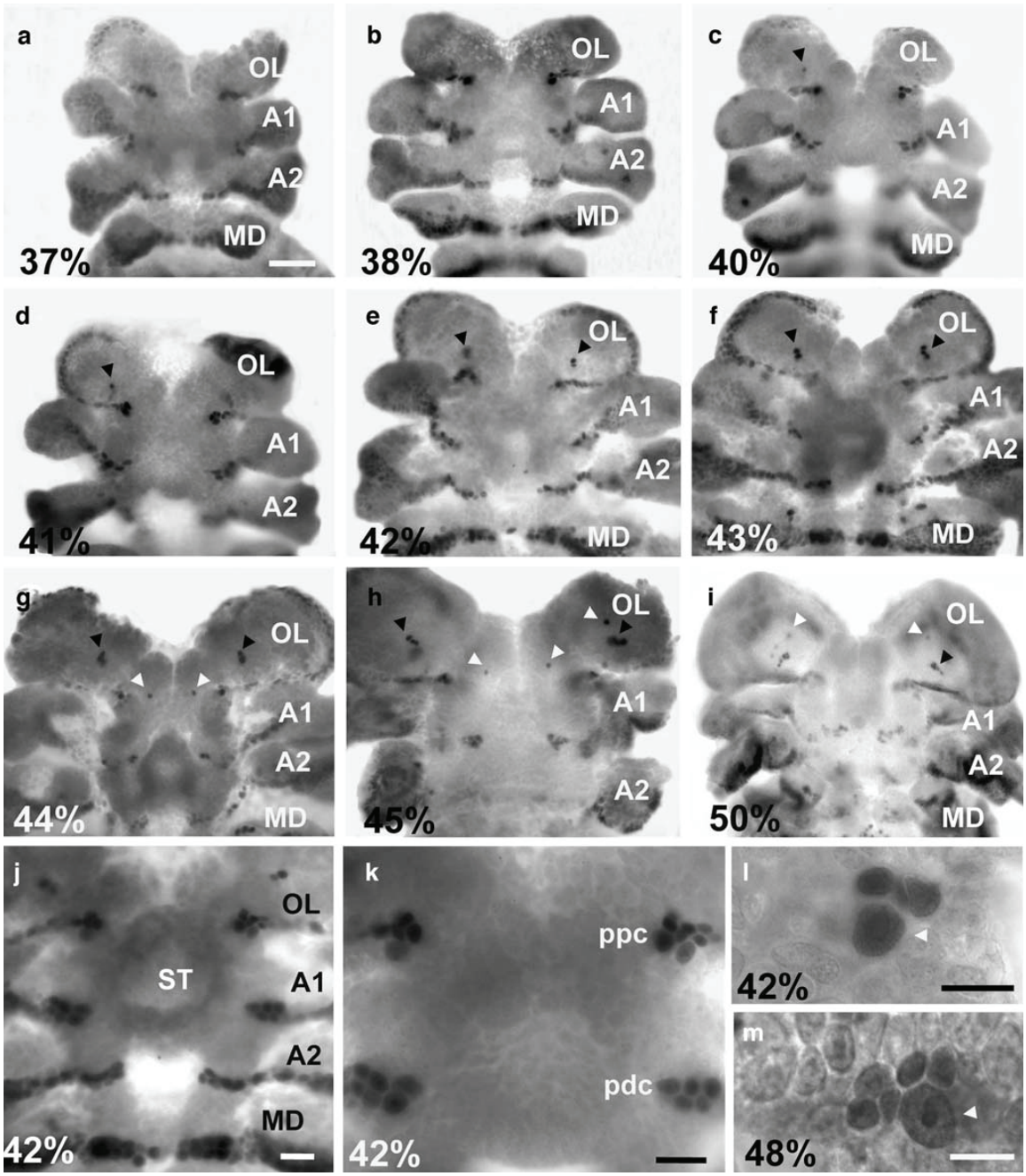


Plate 3.2- a–i: appearance of Engrailed immunoreactivity in the brain from E37% to E50%. Whole mounts of embryos in ventral views. The labeled cells are situated in bilateral symmetrically arranged clusters. In addition to the protocerebral (ppc), deutocerebral (pdc) and tritocerebral (ptc) primary clusters at the posterior neuromere border, between E40% and E50%, a new set of En+ cells gradually appears in the optic anlagen, the secondary head spot cells (black arrowheads in c–i). White arrowheads in g–i label the tertiary head cells that emerge between E44% and E45%. From E45% onwards, the general strength of labeling in the primary protocerebral and deutocerebral clusters diminishes. j, k: higher magnifications of the primary En+ clusters at E42%. l, m: high-power views of the primary protocerebral clusters in two different specimens. White arrowheads label putative En+ neuroblasts. Abbr: A1 antenna one, A2 antenna two, MD mandible, OL optic anlagen, ppc primary protocerebral cluster, pdc primary deutocerebral cluster, ST stomodaeum. Scale bars: a–j 50 µm, k 25 µm, l and m 10 µm.

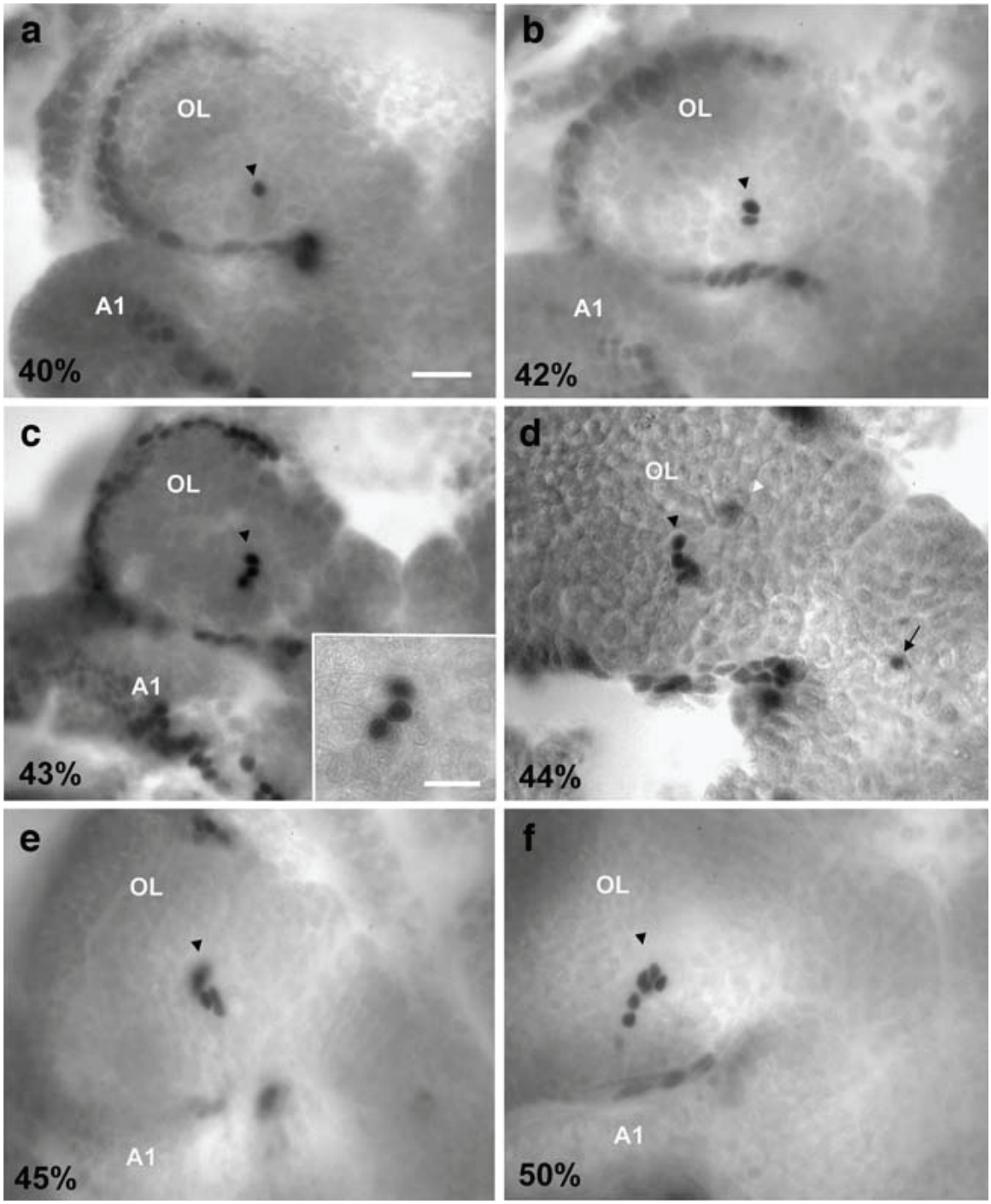


Plate 3.3- Sequential appearance of the secondary headspot cells (shs, black arrowheads) in the optic anlagen from E40% and E50%. a, b: between E40% and E42%, first a single, then a second cell of the shs appears in the middle of the optic anlagen. c, d: between E43% and E45%, three more cells appear just below the first cells and the entire cluster forms a column in many specimens. e, f: by E50% the emergence of the sixth cells completes the group of the secondary head spot cells. The white arrowhead labels a tertiary head cell, and the arrow identifies an En+ cell in the median protocerebrum. Abbr: A1, antenna one; OL, optic anlagen. Scale bar: 25µm (a-f); 10µm (c, inset).

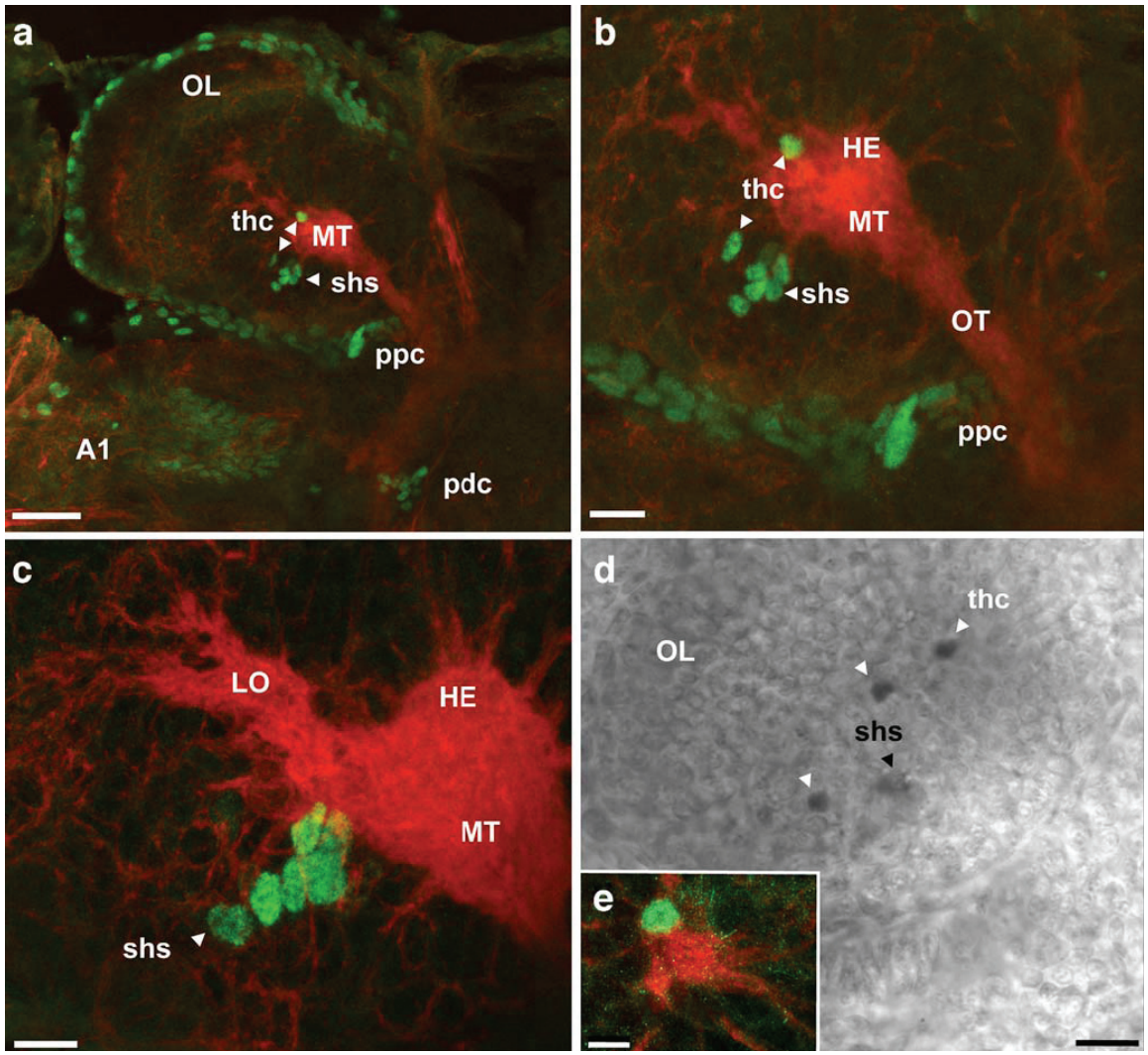


Plate 3.4- a-c: confocal laser scan images of the brain of an E50% embryo, double labelled for engrailed immunoreactivity (green) and phalloidin (actin; red). The secondary head spot cells (shs) are located close to the medulla terminalis/hemiellipsoid body complex (a–c). The tertiary head cells (thc) are located slightly anterior/lateral to the shs cluster (a, b; not in focus in c). d: by E50%, three separate thc have emerged (white arrowheads), which are arranged in an arc-shape. e: higher magnification of a tertiary head cell at E50% to show its close association with the developing optic neuropil. The image is a projection of a seven-image stack (0.44 µm interval) covering a total z-distance of 2.64 µm. Abbr: A1 antenna one, MT medulla terminalis, HE hemiellipsoid body, OL eye anlagen, LO lobula, OT optic (protocerebral) tract, ppc primary protocerebral cluster, pdc primary deutocerebral cluster, shs secondary head spots, thc tertiary head cells. Scale bars: a 50 µm, b and d 25 µm, c 10 µm, e 5 µm.

Chapter 4

A molecular approach

4.1 INTRODUCTION

4.1.1. Molecular phylogeny: comparing aminoacid sequences

The base of molecular phylogenetics (or molecular systematics) is the study of the molecule structure to infer evolutionary relationships between species. Studying phylogeny from the molecular side, by aligning the primary sequences of proteins, comparing the secondary structures of proteins and building phylogenetic trees can be of extreme utility in filling gaps of the phylogeny puzzle (Suarez-Diaz and Anaya-Munoz, 2008).

The first approach for characterizing proteins is the protein sequence comparison: the assumption is that proteins with similar sequences are derived from a common ancestral protein and are called homologous (Gabaldón, 2005). The alignment of multiple sequences aims to place homologous residues of different proteins on top of each other. This is a crucial step for the study of the evolution of proteins because it assumes that all positions in a column of a multiple sequence alignment derive from a common ancestral residue (Gabaldón, 2005). In any point of a given sequence, a base can be found different between organisms and to explain the amino acid substitutions observed. A computer-based approach can be applied in order to calculate the evolutionary distances between all pairs of proteins. The development of algorithms for phylogenetic reconstruction, database search and multiple sequence alignment represents the one of the main contribution of bioinformatics to the study of protein evolution (Gabaldon, 2005). The applications of phylogenetic trees, besides the traditional use in taxonomy, can be multifold: they can be used to "establish orthology and paralogy relationships among proteins, to detect horizontal gene transfers (HGT), gene and genome duplications, positively selected residues, to define strains in epidemiologic studies, to predict functional interactions among coevolving genes or to estimate model parameters and substitution rates" (Gabaldon, 2005).

Not only the structure but also the function of genes and, consequently, fundamental mechanisms may change and evolve trough time. In the last twenty years, comparative embryological studies on the expression of specific genes (homeotic genes and cephalic gap genes) and on morphogenesis have revealed striking similarities between insects,

annelids and vertebrates (Kammermeier and Reichert, 2001). These recent results have suggested a common origin of the basic architecture of the bilaterian brain (Reichert, 2002). In fact, despite the evidence of obvious differences in the neuroanatomy and embryogenesis of a fly and a mouse, we face the evidence of conserved roles of genetic mechanisms that may regulate embryonic brain patterning. Investigating the genetic activity that takes place in cells is one of the main purposes of the molecular biology and localizing the activity of a gene is a powerful tool for studying the development as an indicator of protein function in single cells. Measurement of the expression of a gene can be done by either detecting the final gene product (the protein, by applying immunohystochemical techniques) or by detecting the precursor, typically mRNA, by using the *in situ* hybridization.

4.1.2. *in situ* hybridization

As the name suggests, *in situ* hybridization is a method of localizing, either mRNA within the cytoplasm or DNA within the chromosomes of the nucleus, by hybridizing the sequence of interest to a complimentary strand of a nucleotide probe. The technique was developed by Gall and Pardue (1969) and, independently, by John and colleagues (1969). At that time, radioisotopes were the only labels available for nucleic acids and autoradiography was the only way to detect hybridized sequences. Nowadays, different techniques are available, according to the type of probe or the choice of labeling. For instance, FISH (fluorescence *in situ* hybridization) uses fluorescent probes that bind to parts of the chromosome with high degree of sequence similarity. It is a 20 years technology, applied for the first time by Bauman and colleagues (1980) and since then continuously improved. Fluorescence microscopy can be used to find out where the fluorescent probe is. FISH can also be used to localize specific mRNAs within tissue samples and to study patterns of gene expression within cells and tissues.

in situ hybridization is very popular in evo-devo approach and molecular systematic because it allows the comparison of different pattern of expression to speculate about evolution. As mentioned above, enhancers are thought as primary sites for developmental evolution and studies based on *in situ* link different expression pattern with mutations in enhancers. The fact is that the technique has its own limit, such as the impossibility to

detect post-transcriptional events that alter mRNA stability or changes in splicing that affect the sequences detected by the probe (Alonso and Wilkins, 2005).

Nevertheless, the technique is considered useful in neurobiology where the tight regulation of gene transcription is crucial for the correct development of the brain. By *in situ* hybridization, it is possible to localize a specific gene expression in a specific cell types in a specific region of the brain and to monitor the changes of the expression occurring during development (Islam, 2007). *in situ* hybridization allows to take a snap-shot of the whole brain and understand how synaptic plasticity of cells from different regions in the brain work simultaneously to mediate processes such as learning and memory (Islam, 2007).

4.1.3. Transcription factors

Processes like regionalization, patterning and identity during embryogenesis depend on gene families such as *Hox* genes, the cephalic gap genes and *Pax* genes. They encode for transcription factors that modulate the cascade of events that regulate these crucial processes. The structure of a transcription factor contains domains with specific characteristics: the DNA binding domain which binds to specific sequences of the DNA (such as promoter or enhancer) and the trans-activating domain to bind to other proteins (Latchman, 1997). The DNA binding domain can show different features in the secondary structure and, thus, it has been grouped in different families such as the helix-turn-helix, the basic-helix-loop-helix (bHLH), and the paired box (Latchman, 1997).

The helix-turn-helix represents the most common motif binding to DNA. In eukaryotes, it characterizes the homedomain. It is made of 60-amino acid and the secondary structure comprises an helix-turn-helix structure in which three alpha helices (instead of two) are connected by short loop regions. The N-terminal two helices are antiparallel and the longer C-terminal helix is perpendicular to the axes established by the first two. It is this third helix that interacts directly with DNA (Latchman, 1997). The homeodomain is expressed by the homeobox, about 180 base pair (bp) long and it binds to the DNA in a specific manner. However, the specificity of a single homeodomain protein is usually not enough to recognize the target genes. Homeodomains act as complexes with other

transcription factors which have higher specificity than the single homeodomain (Latchman, 1997).

4.1.4. *Pax 6*, *orthodenticle*, and *atonal*: the evolution of the nervous system

4.1.4.1. *Pax6*: the structure

Pax6 is a member of the Pax-class of transcription factors that have in common the DNA-binding domain (the paired box) which contains a conserved 128-amino-acid DNA-binding 'paired' domain, named after the prototypical *Drosophila paired* gene (*Prd*) (Xu et al., 1999; Fig. 4.1 and 4.2).

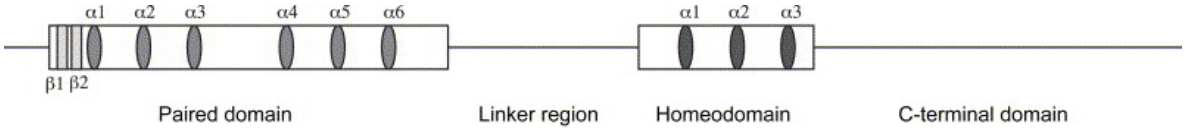


Fig. 4.1- Schematic diagram of the Pax6 protein structure. The paired domain is made of two , (ß1 and ß2) followed by two domains, respectively called the N-domain and the C-domain. Both domains consist of 3 alpha helices (α1-2-3 and α4-5-6) that form the typical helix-turn-helix motiv, able to bind to the major grooves of the DNA. The homeodomain consist of 3 alpha helices, α2 and 3 form an helix turn motif which binds to DNA. The C-terminal of the Pax6 structure is a transactivation domain (from Callaert et al., 2006).

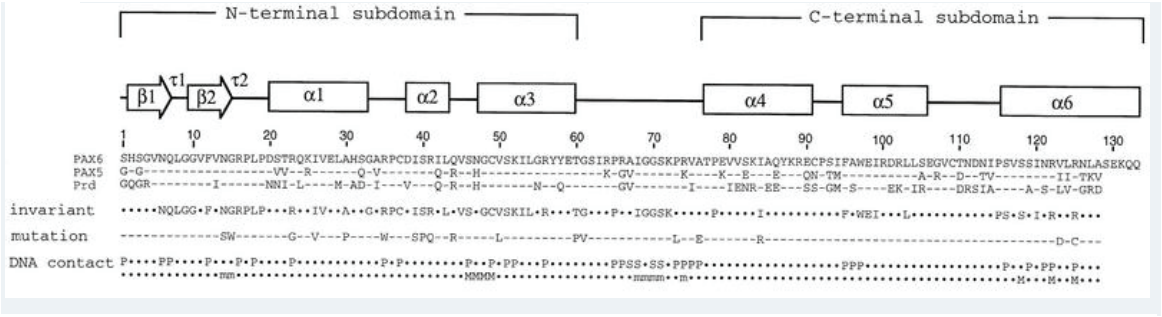


Fig. 4.2- Schematic diagram of the paired domain and binding sites. The paired domain is compared with sequences of paired domains of Pax*5* and Paired domain proteins (Prd) of *Drosophila*. (from Xu et al., 1999). Abbr: α1-6, alpha helices; ß1 and ß2, beta-sheets.

Pax-6 encodes proteins which contain a paired domain, a linker region of variable length, a 60-aminioacid-long homeodomain, and a C-terminal proline-serine-threonine (PST)–rich region (Callaert et al., 1997). In particular, the paired domain is made of N- and C-terminal subdomains called PAI and RED, separated by a linker (Jun and Desplan, 1996).

The N-terminal PAI domain contains two antiparallel β-sheet and three alpha helices: helices 1 and 2 in the N-terminal PAI domain are packed in an antiparallel arrangement. Helices 2 and 3 form a helix-turn-helix unit. A similar arrangement is present in the C-terminal RED domain (Fig. 4.2). The primary sites of DNA interaction occur by the so-called "recognition" helices α3 and α6. Sequence comparisons show that the N subdomain is relatively well conserved among Pax proteins, less similarity is seen in the C subdomain (Xu et al., 1999). Unlike the HOX (HOmeo boX) family of homeobox genes, important during embryonic morphogenesis, *PAX* genes are not clustered but are dispersed throughout the genome (Mark et al., 1997). *Pax-6* genes have been isolated from species of different *phyla.* The first ones were from vertebrates: humans, mouse, and zebrafish. The first invertebrate *Pax-6* gene was isolated from *Drosophila*, *eyeless*, (Quiring et al., 1994), and some years later a second *Pax6* gene, named *twin of eyeless* (*toy*), was also cloned (Czerny et al., 1999). *Pax-6* homologues from the flatworm *Dugesia tigrina*, the ascidian *Phallusia mammillata*, the ribbonworm *Lineus sanguineus*, the squid *Loligo opalescens*, the sea urchin *Paracentrotus lividus*, the nematode *Caenorhabditis elegans* have been also isolated (see review in Callaert et al., 1997). More recent examples are from polychaete worm (Arendt et al,, 2002), jellyfish (Kozmik et al., 2003), *Glomeris marginata* (Myriapoda) (Prpic, 2005) and the horseshoe crab *Limulus polyphemus* (Blackburn et al., 2008). The length of Pax6 proteins varies between 422 aminoacids in humans to 838 in *Drosophila* (Eyeless). Usually in invertebrates, Pax-6 proteins are longer, the linker region of Eyeless, for example, is three times as long as the vertebrate linkers (Callaert et al., 2006).

4.1.4.2. *Pax6*: the function

Data of *Pax6* homologues from species of different *phyla* suggest *Pax6*-dependent mechanisms that may represent a common background in the development of the nervous system in the animal kingdom (Callaert et al., 1997). The best known function of *Pax6* is the central role in eye development in species such as flatworms, *Drosophila*, mouse and humans (Callaert et al., 1997). By physiological, biochemical and molecular studies it has been suggested that all compound eyes in arthropods have a common evolutionary origin (Paulus 1979, Callaert et al. 2006, Harzsch and Hafner 2006). *Pax6*, thus, may be also

informative in terms of evolution of Arthropods due to the similarities in the ommatidia layout and the conserved molecular patterning proposed (Callaert et al., 2006).

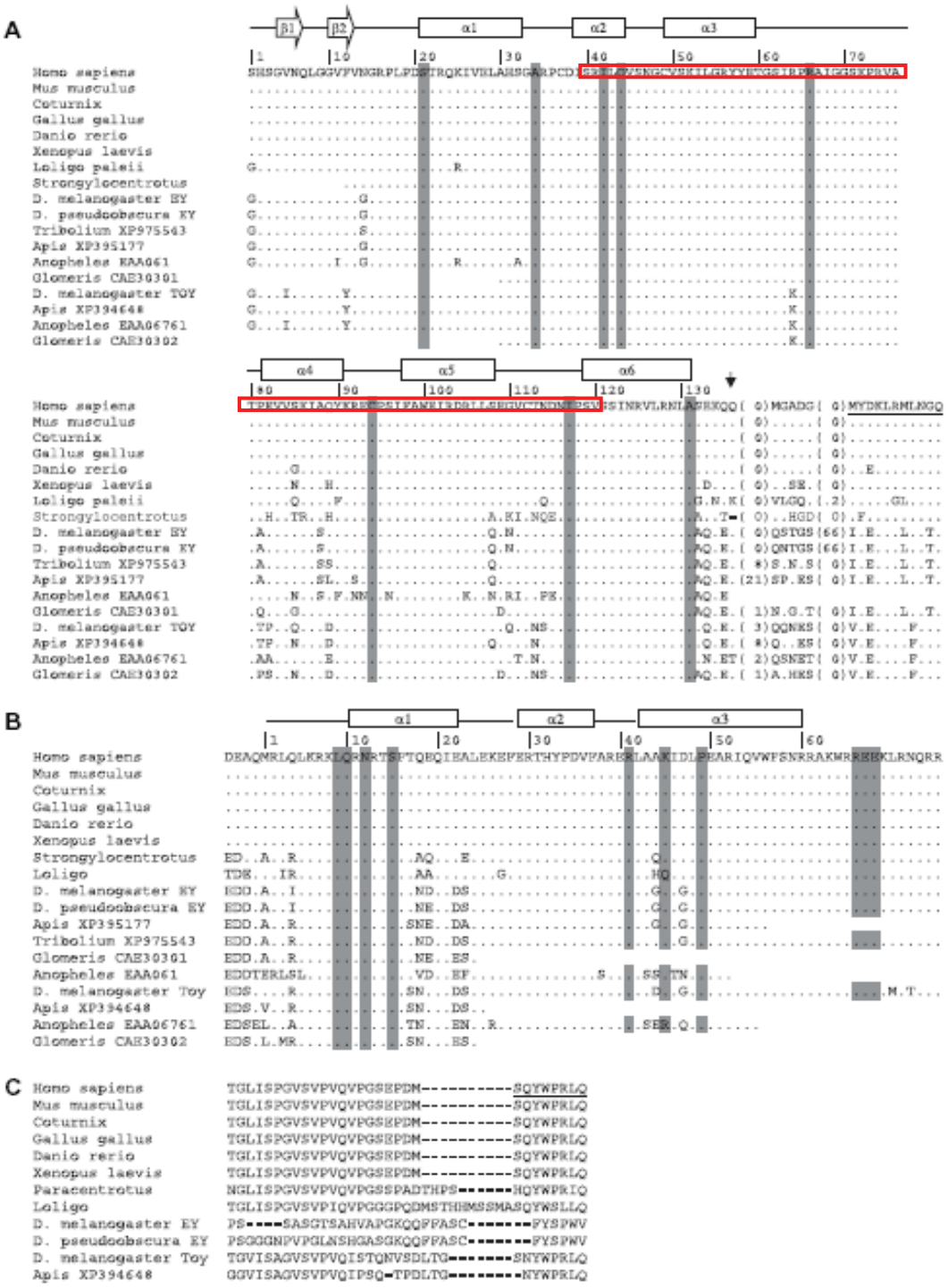


Fig. 4.3- Aminoacid sequence alignment of Pax6. A: The paired domain of Pax6 of vertebrates and invertebrates. The secondary structure is shown above the figure. The sequence highlighted in red represents the part of the gene isolated in the Marbled crayfish. B: The homeodomain of Pax6 of vertebrates and invertebrates. C: C-terminal domain of Pax6 of vertebrates and invertebrates. (modified from Callaert et al., 2006). Abbr: α1-6, alpha helices; ß1 and ß2, beta-sheets.

Current data show a strong conservation of the primary sequence, at least the DNA-binding paired and homeodomains (Fig. 4.3), but its functions have been shown to be not restricted to the visual system morphogenesis. Catmull and colleagues (1998), for example, argued that eye specification might have not been the ancestral role of *Pax6*. Studies on the Cnidaria (the Anthozoa) ended up in a model for evolution of the *Pax* classes which proposes the capture of a homeobox by an ancestral *Pax* gene. This allowed the change in function from cell-fate specification to anterior patterning and ultimately in central nervous system patterning and eye specification (Catmull et al., 1998). In *Drosophila melanogaster*, there are two related *Pax6* genes, *eyeless* (*ey*) and *twin of eyeless* (*toy*). Kammermeier and colleagues (2001) showed that these two related genes have a clear distinct spatiotemporal expression patterns. They are thought to derive from a common origin, by gene duplication (Czerny et al., 1999), but apparently they diverged after duplication because they show different expression and function. During early embryogenesis, they are co-expressed but, whereas *ey* is expressed in the developing ventral nerve cord, *toy* is expressed in the brain. Later in development, both *ey* and *toy* are expressed in specific different subset of neurons (Kammermeier et al., 2001). In particular, *eyeless* is essential in neurons of the protocerebrum, in the optic lobes, the mushroom bodies and the central complex (Callaert et al., 2001). Moreover, *Pax6* has been suggested to play a role in neurodevelopment, cell proliferation and differentiation in the developing cerebral cortex of mouse as well as migration and adhesion at various sites in the CNS (Davis and Reed., 1996), and in general, in sensory development and disease (van Heyningen and Williamson., 2002). In mammalians, *Pax6* contributes to both embryonic and adult neurogenesis as a multifunctional regulator: it is critical for neurogenesis in the human fetal brain and promotes neurogenesis in human neural stem cells (Mo and Zecevic 2008, Kallur et al 2008). Also in mice, an involvement of *Pax6* in the neurogenic role of radial glial cells has been demonstrated, suggesting its function as potent neurogenic gene (Heins et al., 2002).

In some other species, *Pax6* expression has been observed in structures other than those summarized above and this may be the sign of new acquired functions of *Pax6* (Callaert et al., 2001). For example, in jellyfish (Kozmik et al., 2003), polychaete worm (Arendt et al., 2002) and Amphioxus (Gilardon et al., 1998) visual system *Pax6* is not expressed. In

jellyfish *PaxB* replaces *Pax6* function, as well as *So* in adult *Platyneris*. A recent study on the horseshoe crab *Limulus polyphemus* (Blackburn et al., 2008), showed that multiple splice products and protein isoforms characterize *Pax6* gene activity. Furthermore, *Pax6* is not expressed in the developing eyes but in the brain and the ventral cord and, together with *atonal*, in the lateral sense organ, a structure whose function is not known yet (Blackburn et al., 2008).

4.1.4.3. *atonal*

atonal belongs to the family of basic helix-loop-helix (bLHL) transcription factors involved in neurogenesis, myogenesis, cell proliferation, tissue differentiation, and other developmental processes (Morgenstern and Atchley, 1999). *atonal* gets its name from the disruptive effects the gene's mutation has on differentiation. The first bHLH motif was first identified by Murre and colleagues (1989): it comprises of about 60 aminoacids and it is made of a small conserved basic domain necessary to bind to DNA by a consensus hexanucleotide ‘‘E-box’’ (CANNTG), and by two helices to form heterodimers with other bHLH proteins. The members of the bHLH superfamily have been classified into different families such PAS, HES, USF, Achaete-scute (AS-C), Mesp, Hand, p48, Shout, Arnt and Atonal (Voronova and Baltimore, 1990). In *Drosophila*, these proteins act as proneural genes which mean that they are required for specifying neuronal identity and differentiation (Ben-Arie et al., 1996. Fig. 4.5). During neurogenesis they act together with specific cofactors and enhancers that provide the molecular context for gene regulation (Powell and Jarman, 2008). In *Drosophila*, *atonal* plays a role in the peripheral nervous system (PNS) development (mechano- and photoreceptors) and it is expressed in the ectodermal proneural cells (Ben-Arie et al 1996, 2000). The chordotonal neurons are among the sensory elements (they provide proprioception and vibration sense) of *Drosophila* that do not require the proneural genes of Achaete-scute to function, but they need the action of *atonal*, that behaves in many ways like the genes of AS-C (Jarman et al., 1995). The *Drosophila* sequence is evolutionarily conserved; it shares similarity within the bHLH domain with *Tribolium castaneum* (the red flour beetle), *Fugu rubripes* (puffer fish), chicken, and human (Ben-Arie et al., 1996). The closest known homologous is the mouse atonal homolog 1 (*Math1*) which shares 82% aminoacid similarity in the

bHLH domain and 100% conservation of the basic domain, essential for target specificity (Ben Arie et al., 2000). Furthermore, it was shown that not only the sequence but also the gene expression pattern was similar between *atonal* homologs suggesting a similar pathway for early development of the nervous system in insects, birds and mammals (Ben-Arie et al., 1996). However, more recent comparisons showed that in vertebrates they do not have such a neural determination function, but they control the decision made by neural precursors to generate neurons and not glial cells (Vervoort and Ledent, 2001). In mouse, although the high degree of sequence conservation, differences in the expression pattern (*ato* is expressed in the PNS, *Math1* in the CNS) have been reported by Ben Arie and colleagues (2000). Moreover, a recent study on *Limulus polyphemus* (Chelicerate) (Blackburn et al., 2008) showed that *atonal* may not be included in the lineage comprising both *Drosophila atonal* and the vertebrate homologous gene. Adding the fact that *Lp atonal* is expressed only in the lateral sense organ (whose function is not known yet), findings suggest a different embryonic development of this species compared to other arthropods (Blackburn et al., 2008).

4.1.4.4. *orthodenticle* and *empty spiracle*: the anterior head

In *Drosophila*, a hierarchic cascade of genes involving gap-, pair rule- and segment polarity genes patterns the embryo's body (Fig. 4.5A). Gene products from the maternal genes regulate the expression of the first three groups of zygotic genes (gap, pair-rule and segment polarity genes, collectively called the segmentation genes because they divide the embryo into a series of segments along the anterior-posterior axis (Klug and Cummings, 2003). In *Drosophila*, the cephalic gap genes include *ocelliless*, often called *orthodenticle* (*otd*), *empty spiracles* (*ems*), *tailless* (*tll*), *buttonhead* (*btd*) and *sloppy paired* (*slp*). The protein OTD is made of 670 amino acids and it contains a homeodomain which is paired-group-like, although OTD lacks the paired domain (Finkelstein et al., 1990). *otd* is essential for defining the antennal segment, which determines both the eye and the antenna, as well as parts of the brain. *empty spiracles*, *buttonhead* and *sloppy paired* are also required to define the antennal segment (Younossi-Hartenstein et al ., 1997). They are expressed at the blastoderm stage of embryogenesis and their expression is needed to specify the anterior regions of the head. Fig. 4.5B).

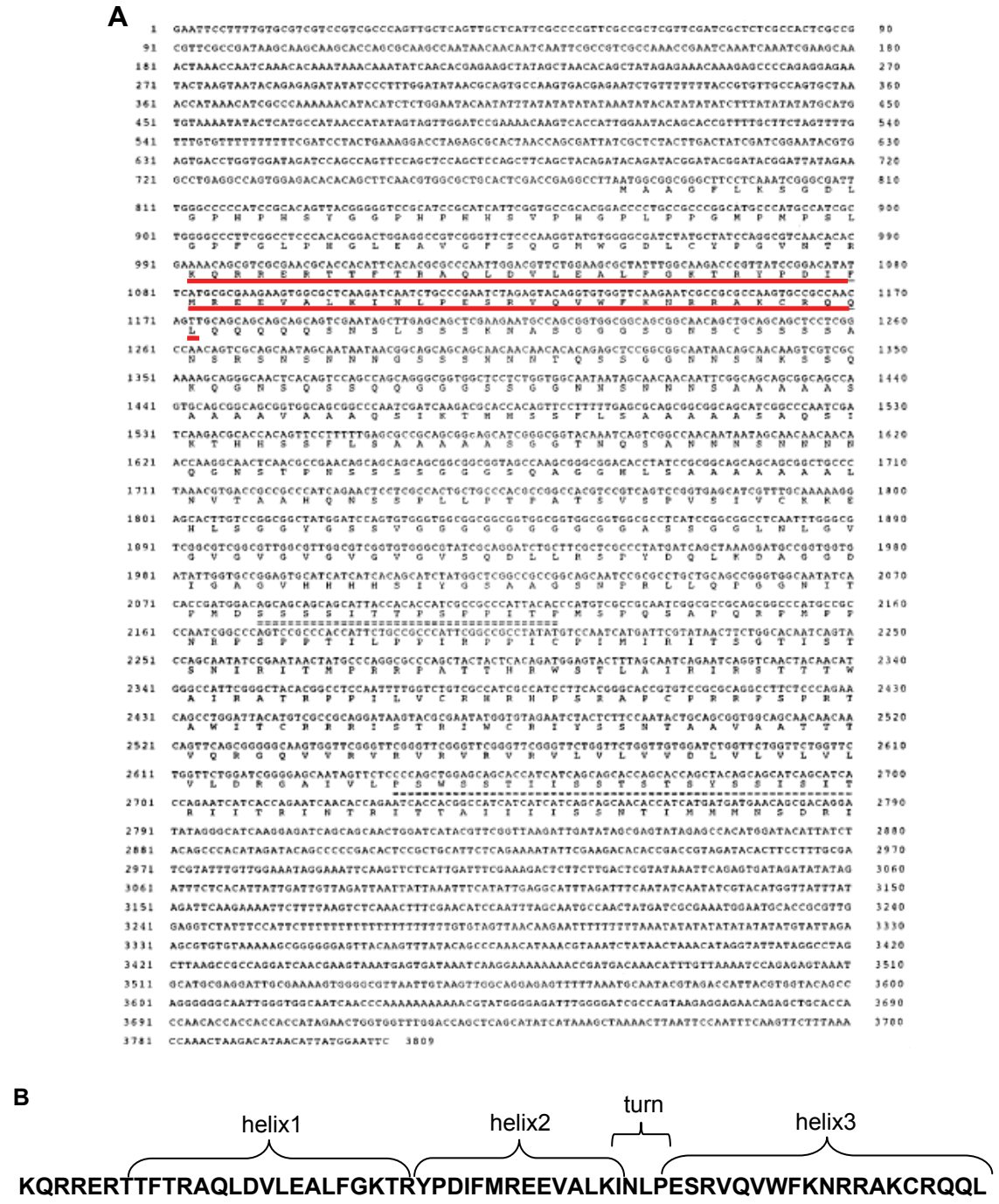


Fig. 4.4- A: the nucleotide sequence of the *Drosophila orthodenticle* cDNA (3.8kb) and the aminoacid sequence of the *otd* gene product. The homeodomain is underlined in red. B: the secondary structure of the *Drosophila* homeodomain of *orthodenticle* (modified from Finkelstein et al., 1990).

These genes also require the presence of Hunchback (*hkb* gene) protein to be transcribed (Simpson-Brose et al., 1994). Together with Bicoid (*Bcd* gene), Hunchback protein acts

at the enhancers of these "head genes" to promote their transcription (Finkelstein and Perrimon, 1990).

A **B**

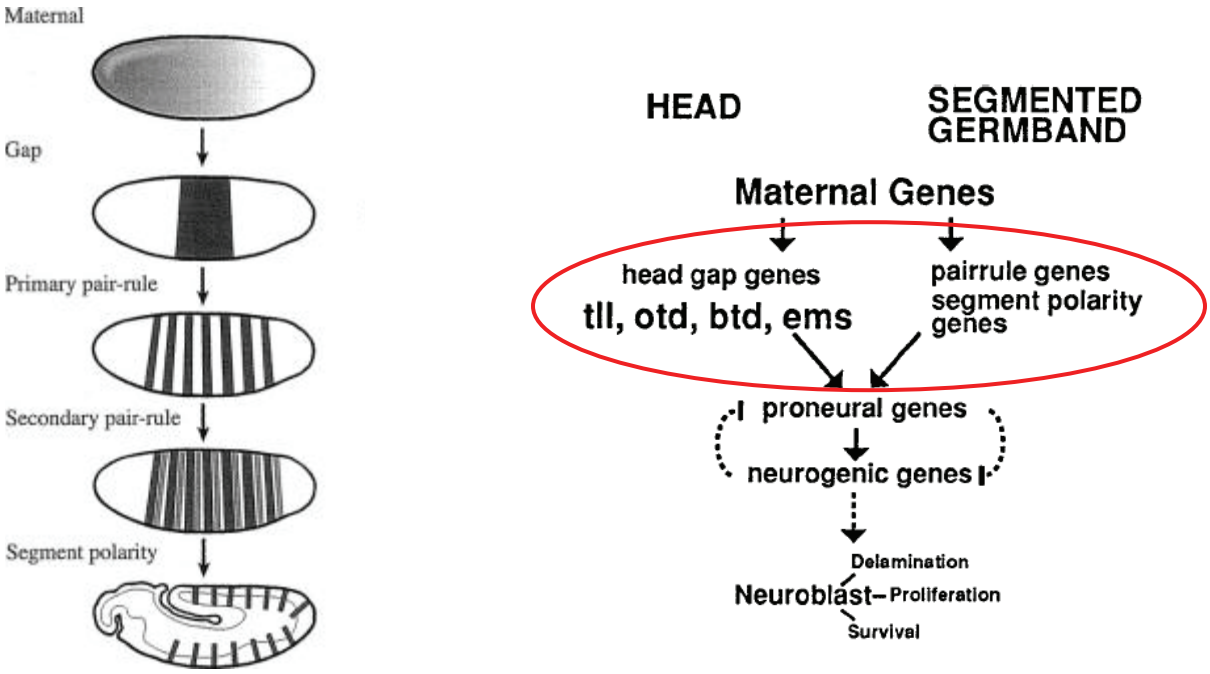


Fig. 4.5- A: Progressive restriction of cell fate during development in *Drosophila*. Gradients of maternal proteins are established along the anterior-posterior axis of the embryo since the beginning of embryogenesis. Zygotic Gap genes divide the embryo into broad regions. The pair rule genes divide the embryo into stripes and segment polarity genes specify the identity of each segment (Klug and Cummings, 2003). B: Genetic control of early neurogenesis by segmentation genes (red circle) which control the expression of proneural genes in *Drosophila* (modified from Younossi-Hartenstein et al., 1997).

In Insects that do not have *bicoid* (which is found only in higher dipterians, like *Drosophila*; McGregor, 2005) anterior patterning is regulated by the maternal *orthodenticle* and *hunchback* (McGregor, 2006). Studies on *Tribolium castaneum* and the wasp *Nasonia vitripennis* suggested that *otd* and *hb* ancestrally performed the role that now is covered by *bcd* in *Drosophila* (Schroder 2003, Lynch et al 2006). Moreover, maternal *otd* is also present in the posterior of *Nasonia* embryos and form a posterior gradient (Lynch et al., 2006). In *Tribolium castaneum*, two otd-related genes were found. Tc *otd-1* early expression resulted similar to that in fly, Tc *otd-2* showed similarities in sequence with the vertebrates counterpart and it is expressed later. Expression pattern of both genes was visible also in the ventral midline and in the mandible (Li et al., 1996).

Recent works reported the findings on other species of Arthropods: two species of Chelicerata, the amphipod crustacean *Parhyale hawaiensis* and the beetle *Tribolium castaneum* (Simonnet et al 2006, Browne et al 2006, Schinko et al 2008). In the chelicerate species, the spider *Tegenaria saeva* and the scorpion *Euscorpius flavicaudis*, it was shown that actually *orthodenticle* and *empty spiracles* are not only expressed in the anterior head but also in a segmental pattern along the anterior-posterior axis during embryonic development suggesting an involvement in different mechanisms at different periods of development. In fact, *otd* and *ems* appeared in segments that will form respiratory organs (Simonnet et al., 2006).

In *Parhyale hawaiensis*, two orthologs show different temporal-spatial expression. *Photd1* shows an expected standard head gap gene expression. *Photd2* expression is not compatible with head patterning because it starts functioning too late to play a role in it (Browne et al., 2006).

To resume these latest results, the *otd* sequence appears quite conserved among arthropods but, contrary to the general and widely accepted notion that *otd* is a marker for head development from insects to mammals, the expression pattern shows peculiarities in non-model organisms such *Tribolium*, *Nasonia* and Chelicerates. Other examples are from *ems*: the late expression of *ems* is similar between insects and chelicerates but for instance in *Tribolium* only part of the deutocerebrum, and not the entire deuto and tritocerebrum seem to depend on *ems* function (Schinko et al., 2008). These types of findings confirm that in most cases concepts about developmental biology of *Drosophila* are not valid for other Arthropods. Non-model animals should become laboratory organisms for comparative purposes and also for increasing the knowledge of their biology. Interesting and useful is, thus, the choice of studying the primary sequence and the expression pattern of such important genes during neurogenesis in the Marbled crayfish.

4.1.5. Crustacea

Developmental biology usually relies on few model organisms, such as yeast, fruit fly, zebrafish and mouse, to study the mechanisms regulating patterning and development. This means that a surprising amount of biological data is obtained by a small number of

species (Stillman et al., 2008). More diverse comparative studies would be needed to wider our knowledge, to understand to which extent processes are conserved among different organisms, and to discover how these processes changed during evolution. So far, comparative developmental studies have been based on the isolation of homologous genes, and comparisons of their sequences and expression patterns among different species. This approach has produced extraordinary amount of information about gene duplication, changes in gene expression, and the evolution of protein sequences during evolution (Pavlopoulos and Averof, 2005). However, limitation has been the characterization of new gene functions in nonmodel species and "has biased comparative studies toward candidate genes that are well studied (in model organisms) and have relatively conserved functions" (Pavlopoulos and Averof, 2005). Lately a change of direction has occurred and the use of non model organisms has become more widespread especially in molecular biology (VanHook and Patel, 2008).

One non-model group of organisms is Crustacea: biologically diverse, ancient, widely distributed and wildly successful (VanHook and Patel, 2008). Crustacea have been often used in physiology, neurobiology and anatomy but only lately researchers have started to apply molecular techniques to these organisms. The problem is that, in order to apply most of the molecular techniques, genome information is strictly necessary but so far only two genomes have been completely sequenced: the one of the branchiopod *Daphnia* (chosen for ecological and historical reasons and for its small genome, 250 Mb) and the amphipod *Jasus slatteryi* (Stillman et al., 2008). In most of the other groups of Crustacea, only mitochondrial DNA sequence have been sequenced, and EST library have been created. The SICB (Society for Integrative and Comparative Biology) Symposium organized in San Antonio, Texas, in 2008, has seen the participation of researchers involved in crustacean genomics projects. The main outcome of the Symposium was the increasing number of projects involved in discovering the DNA of more crustacean species and new models for evolutionary, ecological and genetic studies. Among these, the porcelain crab (genus *Petrolisthes*), the green crab *Carcinus maenas*, the Dungeness crab *Cancer magister* and the amphipod *Parhyale hawaiensis* turned out to be the favorite ones (Stillman et al., 2008).

My project on analyzing the primary sequence and molecular expression of key genes involved in brain development of the Marbled crayfish fits perfectly in this scenario. The project was designed to analyze molecular aspects of embryonic neurogenesis by a PCR based approach, cloning techniques and *in situ* hybridization to understand which genes are involved in neurogenesis in Crustacea. I was successful in cloning crayfish orthologues of the threee genes *Pax6*, *atonal* and *orthodenticle*, all involved in brain development during embryogenesis. Another purpose of the project was the establishment of a protocol for *in situ* applied to the Marbled crayfish which was never developed for such species.

The aim was not only shedding light on the embryogenesis processes from a molecular point of view, but also contributing to increase the knowledge of genetics and molecular evolution of Crustacea and in particular the outstanding laboratory organism Marbled crayfish.

4.2 RESULTS

4.2.1. *Pax6* primary sequence

After the first round of PCR, a faint band was visible in the gel. A nested PCR was then performed with the second set of primers (see Material and Methods, Chapter 6). The PCR assay was the same as for the first PCR, as well as the PCR program. After the nested PCR, I obtained a strong band of about 600 bp. *Pax6* was digested with EcoRI and cloned directly into the vector PGEM-T previously cut with the same enzyme. Size of the insert and sequence of the protein (about 600bp) were confirmed by digestion and sequencing. The sequencing confirmed that the fragment was in frame with the *Pax6* paired domain sequence, in particular with the α2, α3, α4 and α5 sequence (Fig. 4.2).

The alignment of aminoacid sequences shows a higher aminoacid identity with vertebrates (*Rattus n.*, *Tetraodon*, *Mus m.*, *Homo s.*, *Xenopus l.*) than with invertebrates (*Drosophila m.*, *Limulus p.*) although the single values (in %) are not so distant from each other (Fig. 4.6). The least conserved part of the primary sequence is at the level of the alpha helix 4 (in red, Fig. 4.6). The sequence of the helix is not conserved in the Marbled crayfish, while it is utterly identical in all other species. Also, the aminoacid couple "SA" (in green, Fig. 4.6) is exclusive of the Marbled Crayfish and different in other species. It represents part of the turn motif between the helix 5 and helix 6 (see the scheme of the paired domain structure in Fig. 4.2)

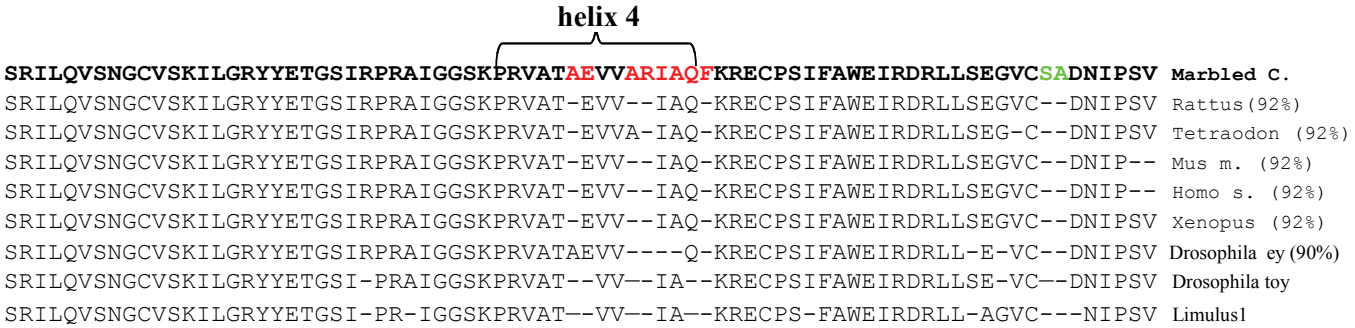


Fig. 4.6- Amino acid sequences alignment of the Pax6 paired domain. The sequence isolated from the Marbled crayfish (in bold) is compared to those of vertebrates and invertebrates. Most of the sequences are highly similar, the region corresponding to the helix 4 shows the highest frequency of differences (in red), as well as the turn motiv between the helix 5 and 6 (SA, in green). Abbr: ey, Eyeless; toy, Twin of Eyeless.

Current data show a strong conservation of the primary sequence of *Pax6* and the present data confirm the current literature. Unfortunately the limited time did not allow performing an adequate RACE PCR to get the full length of the Marbled crayfish *Pax6* gene. That would have been very informative about the characteristic of the sequence of the gene.

Together with the amino acid alignment of *Pax6* genes, the neighbour-joining and the maximum parsimony phylogenetic trees based on paired domain sequences were done, in order to highlight relationships with known sequences of vertebrates and invertebrates stored in the NCBI database.

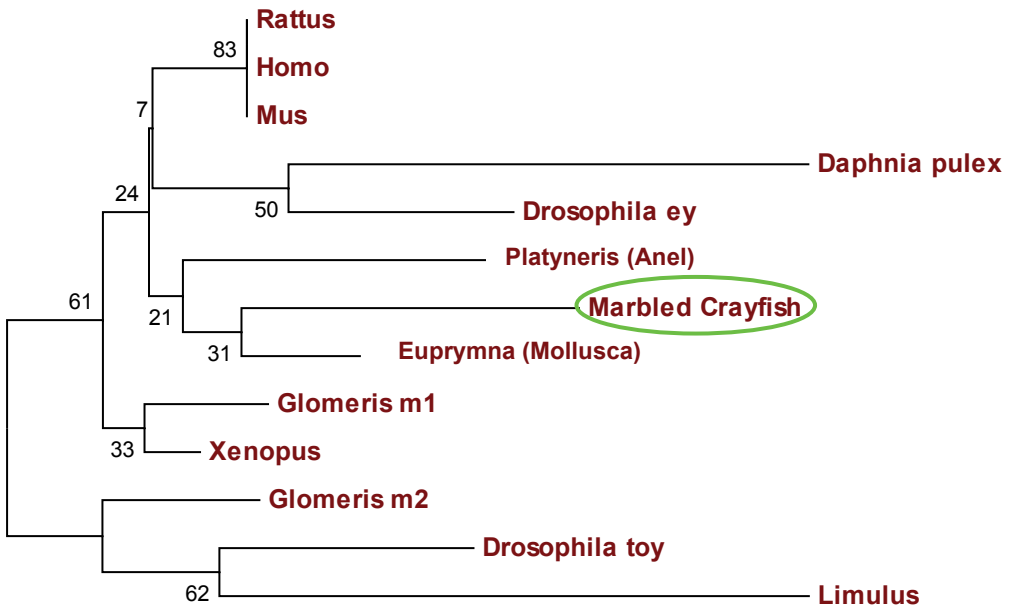


Fig. 4.7- Phylogenetic analysis. The neighbour-joining tree is built from aminoacid alignment of *Pax6* paired domain. The *Daphnia pulex* sequence has been also added. Bootstrap proportions are indicated above the branches (1000 replicates NJ). Abbr: ey, Eyeless; toy, Twin of Eyeless, Glomeris m1/m2, *Glomeris marginata* 1 and 2 (Pax6 homologous).

The neighbour-joining tree (Fig. 4.7) shows a close relation between the Pax6 paired domain of Marbled crayfish and *Euprymna*, a genus belonging to Mollusca. At the same time, our sequence of interest belongs to a lineage that includes *Drosophila* Eyeless and the homologous vertebrates too. However, these relationships are not sufficiently supported by the statistical analysis, because the bootstrap values are too low to be considered reliable. As mentioned above, values should be at least at the 70% value.

Limulus Pax6 is closely related to *Drosophila* Toy and this value may be considered statistically valid (62%), as proved by the Maximum Parsimony analysis performed by Blackburn et al (2008) in which a strong support between the two sequences is shown (bootstrap value: 83%).

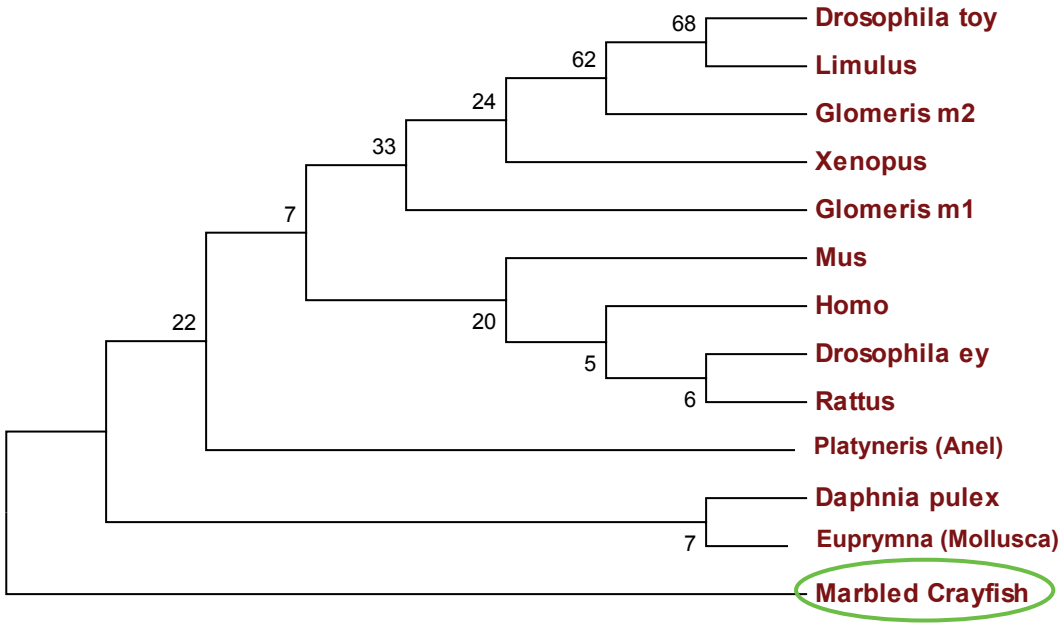


Fig. 4.8- Phylogenetic analysis. The maximum parsimony tree is built from aminoacid alignment of *Pax6* paired domain. Bootstrap proportions are indicated above the branches (1000 replicates NJ). Abbr: ey, Eyeless; toy, Twin of Eyeless, Glomeris m1/m2, *Glomeris marginata* 1 and 2 (Pax6 homologous).

The maximum parsimony shows a similar scenario but the only significant value may be considered, once again, the close relation between the *Limulus* Pax6 and the *Drosophila* Toy (68%) (Fig. 4.8).

4.2.2. *Pax6 in situ* hybridization

The main protocols reported in Chapter 6 (paragraph 6.1.22) were applied to embryos of the Marbled crayfish of different developmental stages. Aim of the experiment was the analysis of the expression pattern of *Pax6* in the brain or in the ventral nerve cord during embryogenesis. Plate 4.1 (see section 4.2.5.) shows embryos of different embryonic stages processed with the in *situ* hybridization. All stages (38%, 42% and 48-50%) do not show any specific signal, at least not the expected one. *Pax6* is mostly involved in eye formation during embryogenesis and a clear staining was likely to be present in the optic

lobes. In all embryos no probe appears to be in the eyes. In the ventral nerve cord, on the contrary, an indication of a signal could be detected, especially if compared to the control. However, due to presence of background and the cuticle all over the tissue, the outcome of the whole mount *in situ* hybridization experiment was not considered up to the standard of an undoubtedly scientific result. A second round of *in situ* analysis was performed on sliced embryos, sectioned by the Vibratome. The thickness was between 50µm and 80µm. By doing the sections, the problem of the covering thin cuticle was supposed to be overcome and the penetration of the probe was supposed to be increased. In spite of these considerations, once again, the outcome of the *in situ* hybridization did not produce the expected result (Pl. 4.2). Unspecific signal is detected almost everywhere suggesting that the protocol is not specific for our species and no conclusions can be drawn out of it.

4.2.3. *atonal*

Same procedure of cloning was applied for *atonal* which appeared as a weak band. A second PCR was run by using the PCR product of the first PCR. Three bands of different sizes appeared: 200bp, 300bp and 600bp. The smallest was the one that, confirmed by the sequencing, corresponded to the bHLH domain of *atonal* gene isolated in *Drosophila* and to the homologous domain of other species.

```
   basic     helix1       loop       helix2
   ┌──┐      ┌──┐        ┌──┐        ┌──┐
AANARERRRMNGLNDAFERLREVVPALGNDRKLSKFEKMAQTMarbled crayfish
AANARERRRMN-LNDAF-RLR-VVP-LGNDRKLSKFE-MAQT Apis mellifera (82%)
AANARERRRMN-LNDAF-RLR-VVP-LGNDRKLSKFE-MAQT Tribolium (82%)
AANARERRRM--LN-AF-RLR---P-LGNDR-LSK-E-MAQT Drosophila m. (75%)
AANARERRRM-GLN-AF--LR-V-PA--ND-KLSK-E-MAQ  Zebrafish (66%)
AANARERRRM-GLN-AF--LR-V-P---ND-KLSK-E-MAQ  Rattus n. (64%)
AANARERRRM-GLN-AF--LR-V-P---ND-KLSK-E-MAQ  Homo s. (64%)
    RER-RM--LN-AF--LREVVP--GNDRKLSK-E--    Limulus (71.4% in 35 aa overlap)
```

Fig. 4.9- Amino acid sequences alignment of *atonal*. The sequence isolated from the Marbled crayfish is compared to those of vertebrates and invertebrates. The most conserved is the basic domain, the HLH regions show more differences in the sequence. Abbr: Math1, Mouse Athonal 1 (Athonal homologous in mouse. See text for explanation).

The sequence corresponds to the motif basic helix-loop-helix which characterizes transcription factors. The closest homolog's cloned so far are insects, *Apis mellifera* and

Tribolium castaneum. The most variable region includes both the first helix and the loop connecting the two helices (Fig. 4.9). As well as seen in *Pax6*, due to the limited time an adequate RACE PCR to get the full length of *atonal* of the Marbled crayfish was not performed. The neighbour-joining and the maximum parsimony phylogenetic trees based on the bHLH domain sequences were made, in order to highlight relationships with known sequences of vertebrates and invertebrates.

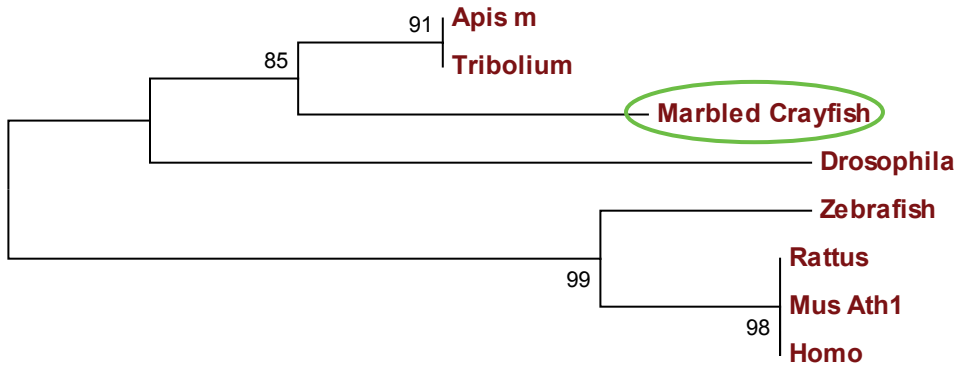


Fig. 4.10- Phylogenetic analysis. The neighbour-joining tree is built from aminoacid alignment of *atonal* bHLH homeodomain. Bootstrap proportions are indicated above the branches (1000 replicates NJ). Abbr: Mus Ath1, *Mus musculus* Athonal 1 (Athonal homologous in *Mus m*. See text for explanation).

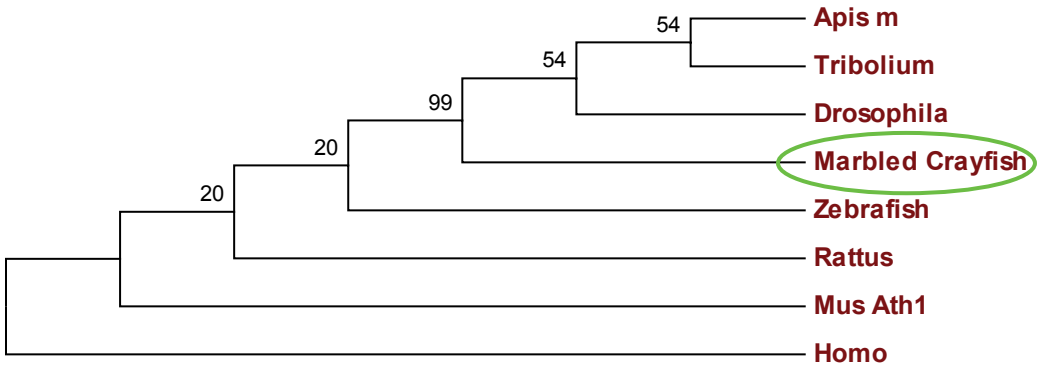


Fig. 4.11- Phylogenetic analysis. The maximum parsimony tree is built from aminoacid alignment of *atonal* bHLH homeodomain. Bootstrap proportions are indicated above the branches (1000 replicates NJ). Abbr: Mus Ath1, *Mus musculus* Athonal 1 (Athonal homologous in *Mus m*. See text for explanation).

The neighbour-joining tree shows a close relation between the *atonal* homeodomain of the Marbled crayfish and Insects (*Apis mellifera* and *Tribolium Castaneum*) (Fig. 4.10). *Drosophila melanogaster*, belongs to the same lineage. These relationships can be considered statistically reliable as the bootstrap values are above minimum value (70%). Same considerations can be done about the vertebrate's sequences which are shown to be

all closely related and statistically significant (Fig. 4.10). The Maximum Parsimony analysis shows a similar scenario, Arthropods sequences of *atonal* are all closely related and statistical values support the relations (Fig. 4.11).

4.2.4. *orthodenticle*

A band of about 100bp was isolated and the sequencing confirmed that was in frame with part of the *orthodenticle* homeodomain. An amino acid sequence alignment together with the phylogenetic analysis was performed (Fig. 4.12).

AQLDVLESLFTKTRYPDIFMREEVALKINLPE	**Marbled crayfish**
AQLDVLESLFTKTRYPDIFMREEVA-KINLPE	Aedes aegypti 96.9%
AQLDVLE-LFTKTRYPDIFMREEVALKINLPE	Apis mellifera 96.9%
AQLDVLEALFGKTRYPDIFMREEVALKINLPE	Tribolium 2 (93.8% identity in 32 aa overlap)
AQLD-LESLF-KTRYPDIFMREEVALKINLPE	Xenopus 93.8%
AQLDVLE-LF-KTRYPDIFMREEVALKINLPE	Drosophila 93.8%
AQLDVLE-LF-KTRYPDIFMREEVALKINLPE	Homo sapiens 93.8%
-QLDVLE-LF-KTRYPDIFMREEVALKINLPE	Artemia franciscana 93.5%
AQLD-LE-LF-KTRYPDIFMREEVA-KINLPE	Tribolium 1 (87.5% identity in 32 aa overlap)
AQLD-LE-LF-KTRY-DIFMREEVALKINLPE	Glomeris marginata 87.5%
-LFTKTRYPDIFMREEVALKINLPE	E. flavicaudis (100.0% identity in 24 aa overlap)

Fig. 4.12- Amino acid sequences alignment of the Orthodenticle homeodomain. The sequence isolated from the Marbled crayfish is compared to those of vertebrates and invertebrates. Abbr: Tribolium 1/2, (Orthodenticle homologous in *Tribolium*. See text for explanation).

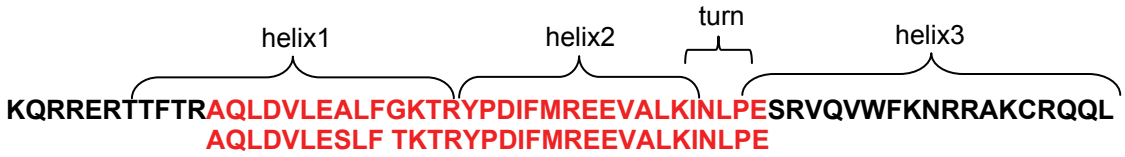


Fig.4.13- Secondary structure of the homeodomain *orthodenticle* gene product. *Drosophila* protein (first line) is compared to the isolated homologous in the Marbled crayfish (red sequence, second line).

The isolated sequence corresponds to part of the helix1, the complete helix2 and the turn motiv. The helix 3 is completely lacking (Fig. 4.13). The closest homologs cloned so far are insects, *Apis mellifera* and *Aedes aegypti*, immediately followed by *Tribolium* (Tc *otd-2*), *Drosophila*, *Xenopus*, humans and *Artemia franciscana*. Previous studies have shown that the similarity of Orthodenticle between species is mostly limited to the homeodomain (about 60 aminoacid) while the rest of the protein can appear even totally different and thus not comparable among different species. In our case, the clone

sequence represents a fraction of the homeodomain in which the most variable region includes the first helix while the rest appears highly conserved (Fig. 4.12).

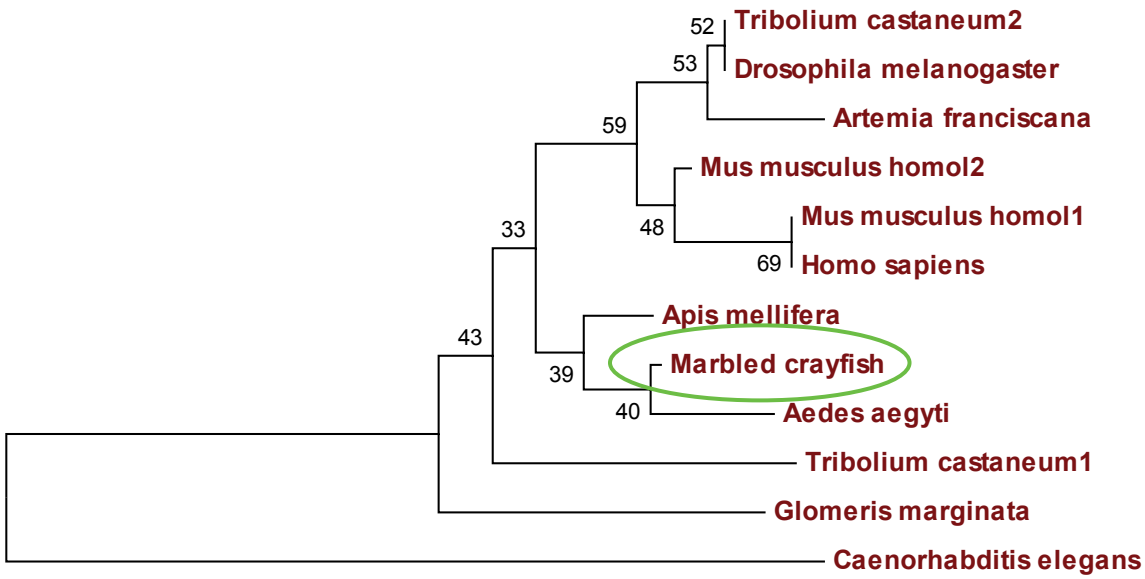


Fig. 4.14- Phylogenetic analysis. The neighbour-joining tree is built from aminoacid alignment of Orthodenticle homeodomain. Bootstrap proportions are indicated above the branches (1000 replicates NJ). Abbr: 1 and 2: Orthodenticle homologous in diverse species.

As well as in *Pax6*, due to the limited time an adequate RACE PCR to get the full length of *orthodenticle* of the Marbled crayfish was not performed. The neighbour-joining and the maximum parsimony phylogenetic trees based on the homeodomain sequences were made, in order to highlight similarities and differences with known sequences of vertebrates and invertebrates in the NCBI database. In the neighbour-joining tree, the sequence of the Marbled crayfish appears isolated by the others, but included in the same lineage of vertebrates and *Drosophila* (Fig. 4.14).

The low values of the Boostrap analysis, however, do not give enough support to this scenario and so the relationships between the sequences can not be resolved in this case. The same situation is described by the maximum parsimony test (Fig. 4.15).

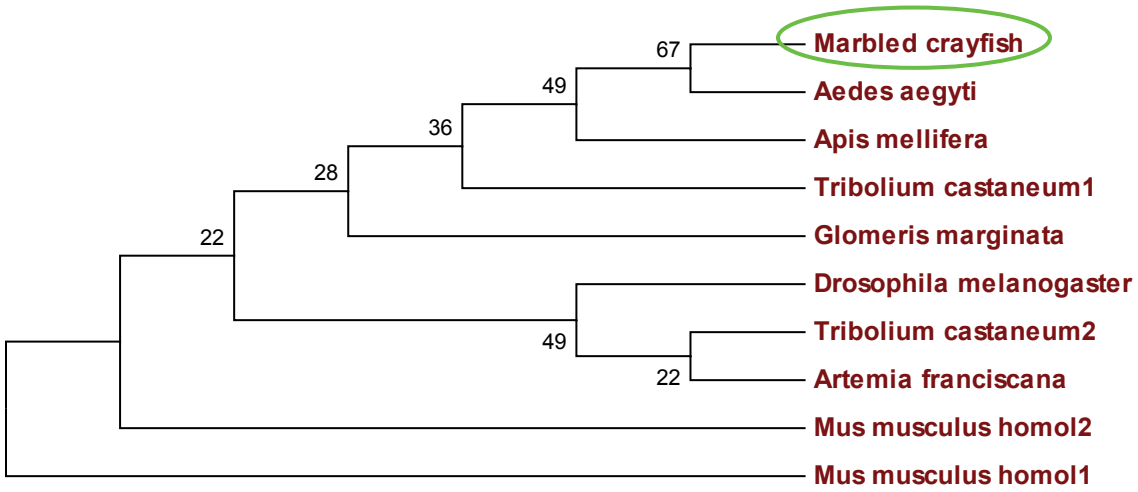


Fig. 4.15- Phylogenetic analysis. The maximum parsimony tree is built from aminoacid alignment of Orthodenticle homeodomain. Bootstrap proportions are indicated above the branches (1000 replicates NJ). Abbr: 1 and 2: Orthodenticle homologous in diverse species.

4.2.5. Plates

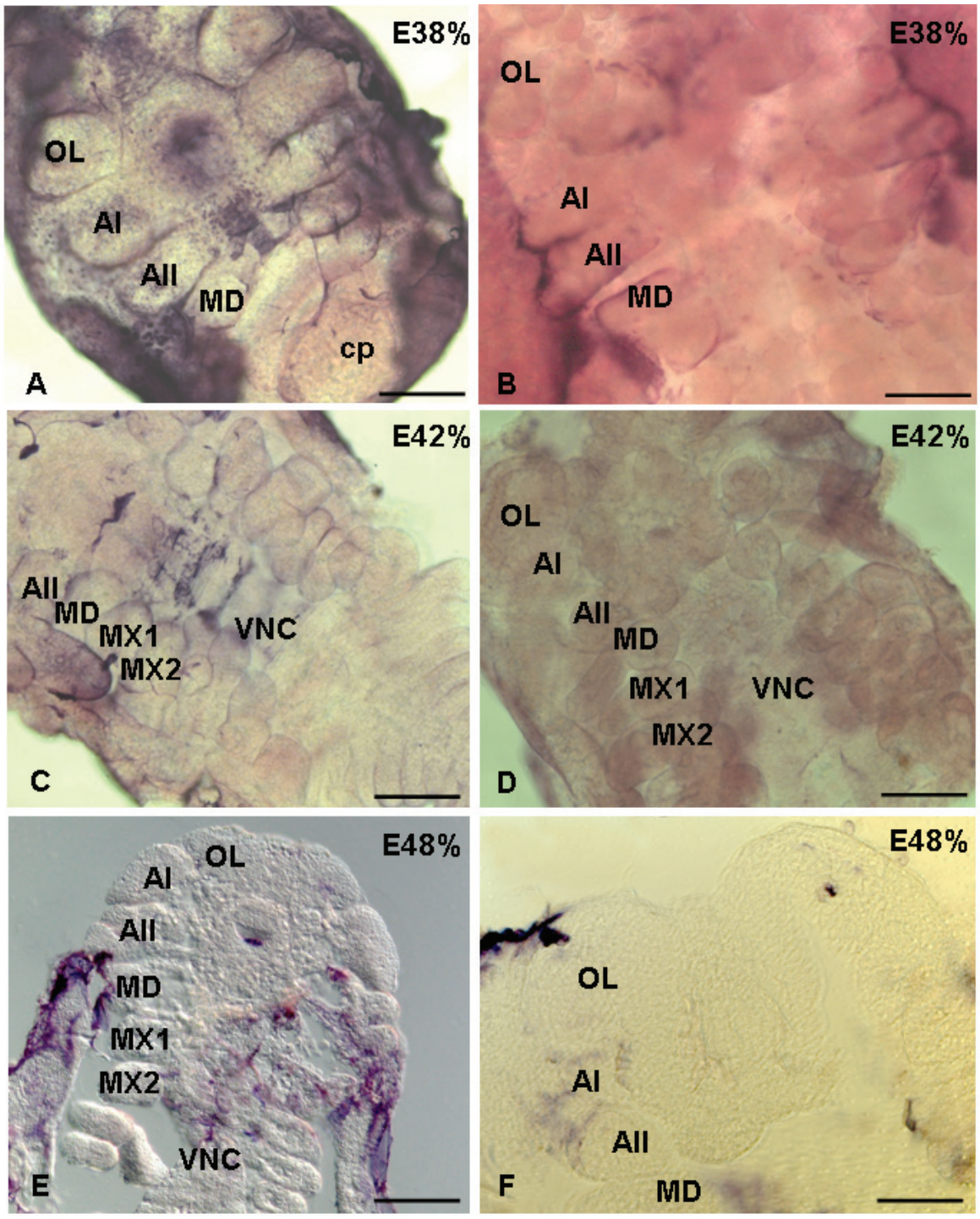


Plate 4.1.- Marbled crayfish embryos at different developmental stages, processed with the *in situ* hybridization. A, C, E: antisense probe. B, D, F: sense probe (the control). Abbr: OL, optic lobes; A1, antenna I; A2, antenna II; MD, mandible; MX1, maxilla I; MX2, maxilla II; cp, caudal papilla; VNC, ventral nerve cord. Scale bar: 50µm

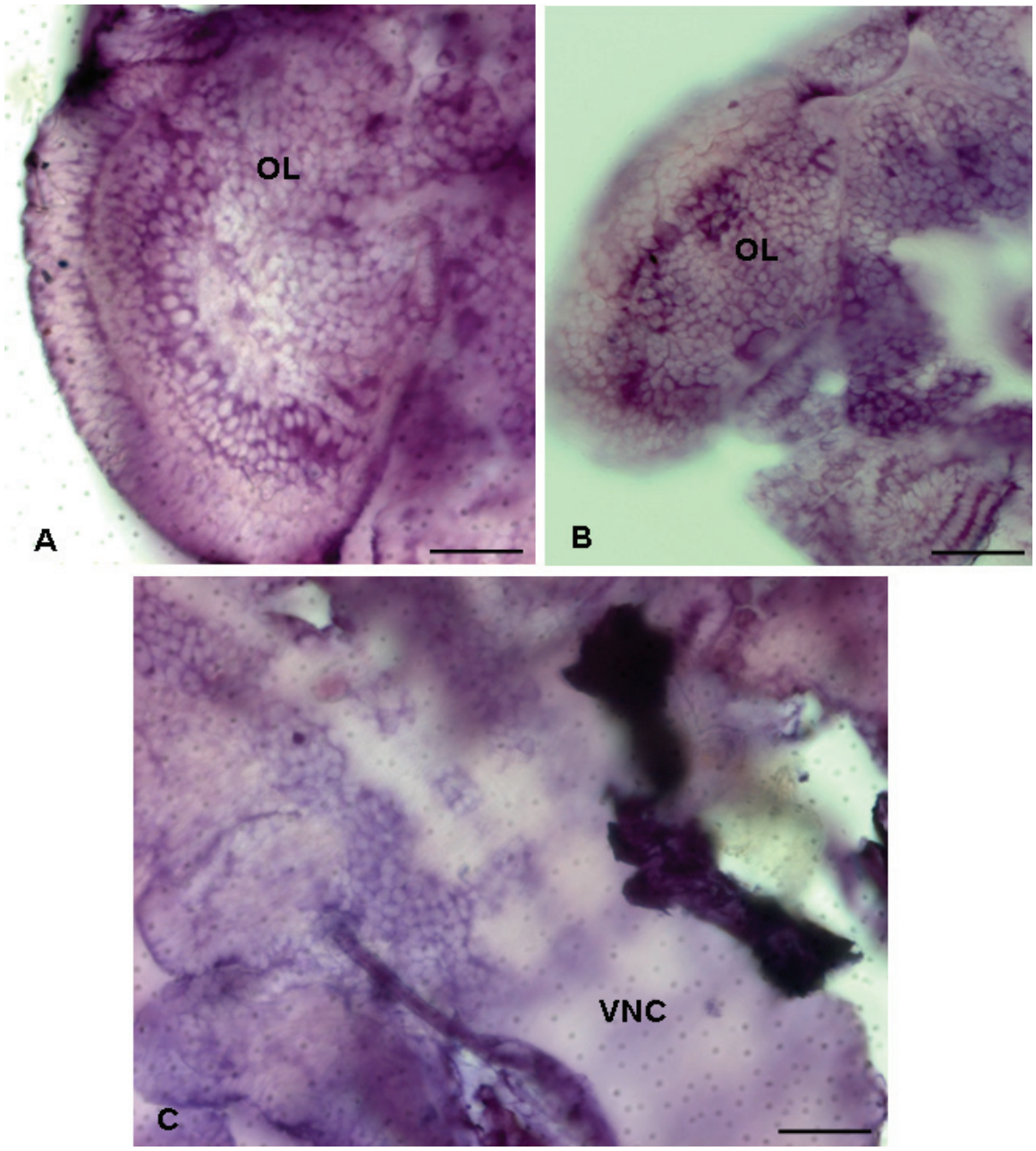


Plate 4.2. - Marbled crayfish embryos at E48%, processed with the *in situ* hybridization. A, C: antisense probe. B: sense probe (the control). Abbr: OL, optic lobes; VNC, ventral nerve cord. Scale bar: 20µm

4.3 DISCUSSION

The main purpose of this project was the analysis of the sequence and the expression pattern of selected genes involved in neurogenesis: *Pax6*, *atonal* and *orthodenticle*. Genes were isolated and cloned into appropriate vectors and the sequencing confirmed that the each sequence was in frame with the expected one. The analysis of the primary sequence and the secondary structure was performed by an alignment and comparison with known sequences of other species. The analysis of the genetic expression was performed by using a *Pax6* probe suitable for the *in situ* hybridization.

4.3.1. Primary and secondary structure

4.3.1.1. *Pax6*

The typical Pax6 protein contains an N-terminal paired domain, connected by a linker region to a paired-type homeodomain. Both domains have DNA binding activity. The highest conservation is at the level of the sequence of the paired domains. For instance, except for the zebrafish Pax6 which has one different aminoacid compare to human (and the two species diverged over 400 million years ago; Glaser et al., 1992), the vertebrate Pax6 proteins share the same paired domain. Concerning the invertebrate Pax6 proteins, the paired domain sequence is identical to the mouse for over 90%, a degree of conservation that approaches that of histones, some of the most highly conserved known proteins (Quiring et al., 1994). The outstanding conservation of the sequence may suggest that changes on it are heavily selected against. According to Hanson and colleagues (1999), each time a mutation arose, it must have had a disadvantageous consequence (for example on eye function, essential for the survival of almost all higher organisms) and would have been eliminated from the population rapidly. The high conservation of the sequence between vertebrates and arthropods is somehow supported by the phylogenic analysis (Neighbour Joining test and Maximum Parsimony, Fig. 4.10 and 4.11) which do not consider the distinction in clades statistically significant. So, we can speculate that during evolution not many changes occurred in the paired domain sequence and therefore the chosen gene shows a high conserved primary structure, as proved by the literature.

The sequence isolated from the Marbled crayfish shows an high identity to many of the known sequences, especially at the level of the N-subdomain (helices 2 and 3). The C subdomain presents more differences: the sequence corresponding to the helix 4 differs in almost half of its structure (Fig. 4.9). The reason may be found in the different functions which characterized the two subdomains. Both the N and C-subdomains have the ability to bind the DNA. However, the N-subdomain usually plays a dominant role in DNA binding of the entire protein due to its higher affinity and specificity (Xu et al., 1999). Moreover, similarities in the amino acid or DNA sequences of their binding sites or in the relative position of residues from the HTH units that make contacts are not present (Xu et al., 1999). This means that in spite of a similar secondary structure and similarity in the folding and docking arrangement, differences in the primary sequences makes also the difference in the functions of the two domains. What Xu and colleagues (1999) infer is that the paired domain may have arisen by gene duplication of a three-helix unit and that similarities in the amino acid sequences of the domains were lost during divergent evolution.

4.3.1.2. *Pax6 in situ*

The protocol of *in situ* hybridization (see Chapter 6, Matherials and Methods, paragraph 6.1.22) was applied successfully on the *Procambarus clarkii* by Hafner and colleagues (2003). The reason why it was chosen for my experiments was, thus, based on the similarity of the *Procambarus clarkii* to the Marbled crayfish (*Procambarus* sp). Nevertheless, either for the different species or for differences in applying the same technique, the method did not work in our case. Another reason could be seen in the length of the probe. Even after the RNA probe has been transcribed successfully from the template, in fact, if the RNA probe is greater than 300-400bps in length it should be hydrolyzed into shorter fragments since the optimal upper length for riboprobes for *in situ* hybridization is 150-200bps. Longer probes may have poor tissue penetration.

In conclusion, a suitable *in situ* protocol for the Marbled crayfish has still to be optimized by adjusting the single steps of the method which has to be tailored for our species.

4.3.1.3. *atonal*

The bHLH domain is characteristic of transcription factors and it is highly conserved throughout the animal kingdom, in fact it was identified in insects and in vertebrates and a high similarity was detected among them. It has been seen that it plays an exclusive role in protein dimerization and recognition of the DNA target, suggesting that it could be the "only functionally significant domain in the proteins" (Ben-Arie et al., 1996). The sequence isolated from the Marbled crayfish shows 100% identity at the level of the basic domain while the most variable region is represented by the loop connecting the two helices. The loop is actually the region that vary in length and that can be used to overall sequence similarity to infer homology relationships (Chen et al. 1996, Ben-Arie et al. 1996). On the contrary, the basic domain determines target recognition specificity and thus is necessary for DNA binding and this may justify the highly degree of conservation of the sequence. An interesting study presented by Ben Arie and colleagues (2000) attempted to demonstrate similar functions of Atonal transcription factor both in *Drosophila* and in the mouse, as a consequence of the high correspondence in the sequence of the bHLH domain and total identity of the basic domain sequence. The result was not the expected one as the two homologs (*ato Drosophila* and *Math1* mouse) showed a discrepancy in the expression pattern. While *ato* is expressed in the peripherical nervous system, *Math1* is expressed in the central nervous system.

Future directions about this gene may be the analysis of the *atonal* expression in the Marbled crayfish to understand its function and involvement in the brain development and to compare the data with those available for *Drosophila* and vertebrate.

4.3.1.4. *orthodenticle*

orthodenticle is characterized by the so called helix-turn-helix homedomain (which comprises 3 helices in eukaryotes) and the cloned sequence corresponds to part of the helix1, the complete helix 2 and the turn. The identity among known sequences is very high: except for *Tribolium* and *Glomeris* that show 87.5% identity, all other species (vertebrates and insects) show more than 93% identity. Because of such high degree of conservation, the low value of the statistical analysis of the phylogenetic trees may find a reasonable explanation. The sequences are so similar to each other because no significant

changes occurred during evolution and nowadays no distinction in clades can be done. The primary structure but also the involvement of *orthodenticle* in patterning the anterior head (thus, the function) has been found quite conserved between arthropods and also vertebrate suggesting a similar mechanism. Once again, studies of the expression pattern of *otd* in the Marbled crayfish may give more support to these hypothesis and shed light on the function of this important head gap-like gene.

4.3.2. Some remarks on phylogenetic analysis

The selection of characters is one of the most important steps to take before approaching any type of molecular phylogeny analysis. Many molecular data can be used as traits: highly conserved genes, proteins or domains, for instance, are considered informative in terms of phylogeny. Searching for the optimal tree, speculating about homology and relationships between systematic and molecular biology and correspondence between them are all current problems in molecular systematic and care has to be taken when discussing such data. DNA can be an excellent tool for inferring phylogeny as DNA evolves by mutations and DNA sequences in different species can show different degrees of homology and thus demonstrate evolution. To estimate phylogeny an estimation of how much a sequence diverges from other species and changes in nucleotide is considered informative in term of phylogeny. However, it can also be argued that it is a mistake to replace a classification based on visible and ecologically relevant characteristics by one based on genetic mutations that may not even be visible in the phenotype. Moreover, cladograms cannot be considered completely true because in any diagram there are a number of possible evolutionary pathways that could produce a similar pattern. The cladogram only illustrates the probability that two sequences are more closely related to each other than to a third one, it does not necessarily clarify the pathway that created the existing relationships. Even more important, an evolutionary tree of a gene family does not necessarily represent the phylogenetic tree of the corresponding species (Brocchieri, 2001).

In spite of these argumentations, cladograms are useful for the formulation of new hypotheses and for shedding light on existing data. The molecular approach to systematics is, in fact, gaining acceptance especially since the gene sequencing has

become easier and cheaper. Also the study of the gene expression (*in situ* technique) is important in developmental approach but it needs to be seen in context. The relationship between genetic and morphological identity can be understood only by considering the developmental pathways (Müller and Wagner, 1996) and always by keeping in mind that genetic identity does not necessarily coincide with morphology identity (Galis, 1996).

4.4 CONCLUSIONS

The outcome of my project is represented by three cloned genes from the Marbled crayfish that, according to previous studies on different species, play a significant role during neurogenesis. The analysis of primary sequences showed a elevated conservation of the three genes sequence, and this is in agreement with the literature that *Pax6*, *atonal* and *orthodenticle* are essential during the development of the brain and for this reason they are highly conserved. The Marbled crayfish does not depart from this view and, thus, the three cloned genes can represent the base for future studies. In order to clarify the function that they explicit in the brain during embryogenesis, an appropriate protocol for *in situ* is necessary. Once the protocol will be established, a clearer picture of the molecular mechanisms underlying the brain formation will become available as well phylogenetic assumptions.

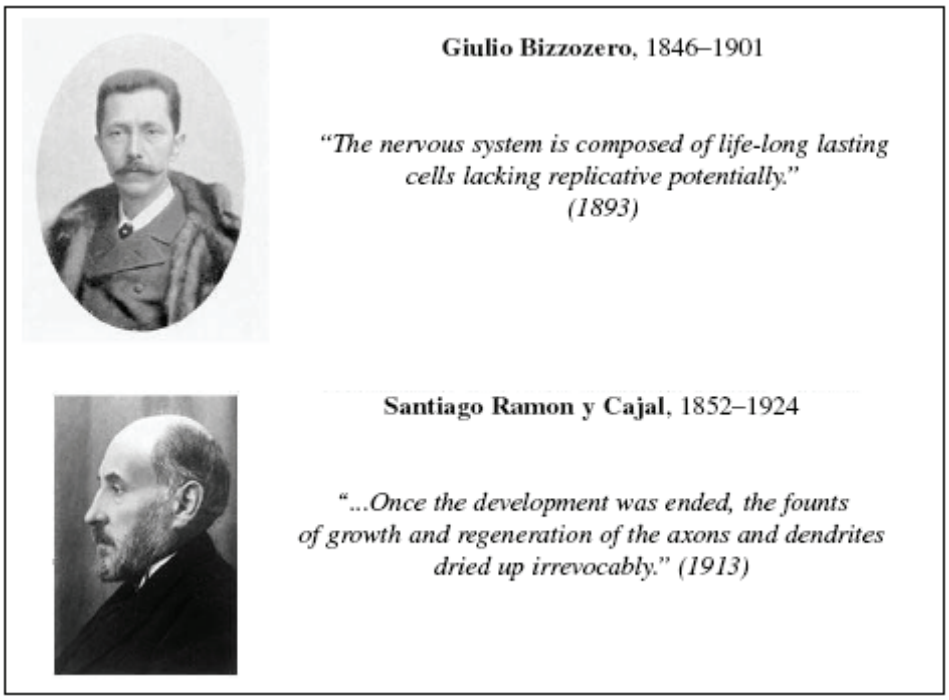
Chapter 5

Adult neurogenesis

5.1 INTRODUCTION

5.1.1. Adult Neurogenesis: the end of the neurobiology dogma

For more than one century, the central belief in neurobiology was that "in adult centers, the nerve paths are something fixed and immutable: everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree" (Santiago Ramon y Cajal, 1913. In: Colucci-D'Amato et al, 2006).



from Colucci-D'Amato et al., 2006

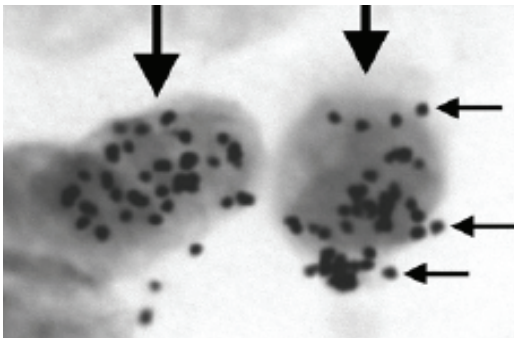
The origin of the dogma lied in the assumption that the elaborate structure of the brain could not allow the addition of new neurons which could alter pre-existing circuits. It was considered an inconceivable idea (Colucci-D'Amato et al., 2006).

More than one conviction was in support of the dogma: patients with brain lesions were not able to recover, chronic diseases such as Parkinson's and Alzheimer could only worsen and the power of techniques was limited (^{3}H-thymidine autoradiography and limited resolution of light microscopy) (Colucci-D'Amato et al., 2006). To use Cajal words, at that time they were "not capable of distinguishing absolutely a multiplying neuroglia cell from a small mitotic neuron" (Santiago Ramon y Cajal., 1913. In: Gross.,

2000). These ideas dominated the scene of neurobiology for decades until in 1962 a young postdoc from the Psychophysiological Department at the MIT (Massachusetts Institute of Technology) published a paper, which still represents a milestone in this field. Joseph Altman reported evidence for "neogenesis of nerve cells" in young and adult rat, suggesting that "new neurons may come into existence in the brain of adult mammals, at any rate in such forms as the rat" (Altman, 1962). Due to the lack of specific molecular markers and more sophisticated technology, his findings were ignored: until the 70´s, the "Developmental Neurobiology" text book (Jacobson, 1970. In: Gross, 2000) was still reporting that "there is no convincing evidence of neuron production in the brains of adult mammal". Other papers from Altman were published during the following years (1963, 1966, 1969. In: Gross, 2000) but, due to the impossibility to clearly distinguish glia cells from neurons by using the light microscopy, they were not accepted by the scientific community. In 1977, Kaplan and Hinds presented results from three months old rats, treated with ^{3}H-thymidine and sacrificed after one month.

The tritiated thymidine technique

Incorporation of tritiated thymidine (3H-TdR) into cellular DNA was and still is used to monitor rates of DNA synthetis and cell proliferation. 3H-thymidine is incorporated into nuclear DNA during the S phase of the cell cycle, and the amount of inclusion is directly proportional to the number of silvergrain overlaying it.Neurons are considered new if the number of overlaying grains is at least 50% of that recorded in maximally labeled nuclei in the same specimen (Rakic, 2002).



By electron microscopy of thinner section, he was able to confirm the identity of neurons. His conclusions stated that "the old concept that the adult mammalian brain is largely static is no longer tenable … Now we have confirmed that growth and plasticity, including neurogenesis and synaptogenesis, can also occur in the mature, unoperated, mammalian brain" (Kaplan and Hinds, 1977).

Beginning of the 80's, Nottebohm applied the same technique (^{3}H-thymidine) to songbirds and he discovered that a neuronal turnover occurred also in the brain of these animals (Goldman and Nottebohm, 1983). Like Kaplan, he used the electron microscopy to identify labeled synapses and he coupled microscopy with neurophysiological recordings (Burd and Nottebohm, 1985). Later on, other contributes from Nottebohm's studies will be the discovery of the influence of environment, experience, learning and hormonal changes on neurogenesis (Nottebohm 1984, Lipkind et al. 2001). In spite of these first evidences and accumulating data on neurogenesis in the brain, again the dogma did not break down. These studies were viewed as irrelevant to the primate or the mammalian brain (see Gross, 2000). In 1985, Pasko Rakic, from Yale University, presented his results on Primates revealing that "not a single heavily labeled cell with the morphological characteristics of a neuron was observed in the brain of any adult animal". Moreover, "minimal radioactivity was occasionally observed over a neuron (…). It is reasonable to assume that the presence (..) of slightly more grains than the background number may reflect a technical artifact". The conclusions were that the brain of primates is "uniquely specialized in lacking the capacity for neuronal production once it reaches the adult stage" and it "requires a stable set of neurons to retain acquired experiences in the pattern of their synaptic connectivity" (Rakic, 1985).

In the 90's, the improvement of the techniques made a significant step towards the acceptance of adult neurogenesis. The introduction of the synthetic thymidine analogue BrdU (5-bromo-3'-deoxyuridine) to mark proliferating cells in the S-phase of the cell cycle allowed the use of immunohistochemistry instead of autoradiography. New neuronal markers became available as well as the confocal technology and the combination of the three techniques was used by Kuhn and colleagues (1996) to show the neurogenesis in the dentate gyrus of rat.

At the end of the 90's, adult neurogenesis was eventually proved to occur in primates (Gould et al. 1999, Kornack and Rakic 1999) and finally in the hippocampus of humans, treated with BrdU (Ericksson et al. 1998).

In 2004, Bédard and Parent demonstrated the evidence of newly generated neurons in the human olfactory bulb which "is a site of active cell proliferation as well as a recipient for

migrating neuroblasts that mature and express morphological and chemical phenotypes typical of granular and periglomerular interneurons".

To conclude, it has been seen that adult neurogenesis is widespread in the animal kingdom, however in each species it occurs in specific areas brain and few neuronal types are subject to it. Here a summary of the main studies on vertebrates: elasmobranchs (Leonard et al., 1978), teleost fishes (Raymond and Easter 1983, Zupanc 2006), amphibians (Polenov and Chetverukhin, 1993), reptiles (Font et al., 2001), songbirds (Goldman, 1998), and mammals, so far the best analyzed group (reviews: Gross 2000, Christie and Cameron 2006). Across various rodent and primate species including humans, adult neurogenesis was demonstrated in two brain areas, the subventricular zone/olfactory bulb and the dentate gyrus of the hippocampus (Eriksson et al. 1998, Kornack and Rakic 2001, Christie and Cameron 2006).

5.1.2. Adult Neurogenesis in Invertebrates

A similar dogma on inflexibility of the brain was formulated for invertebrates, due to their rigidly programmed development of the nervous systems.

> "The detailed similarity of nervous systems in individuals of the same species of invertebrate makes it somewhat unlikely that competition between populations of nerve cells, feedback and prolonged periods of neural malleability play an important role in establishing patterns of invertebrate neural connections"
> (Easter, Purves, Rakic and Spitzer, 1985).

Nevertheless, compare to vertebrates, invertebrates could not vaunt such similar history of studies and consideration. So far, most of the attention has focused on the two major invertebrates groups: insects and crustaceans and not a single study has been done for instance on the other classes of arthropods, chelicerates and myriapods, as well as in groups like annelids, nematodes and flatworms (Lindsey and Tropepe, 2006). Same situation is valid for mollusks and echinoderms which did not receive much interest from researchers (Lindsey and Tropepe, 2006).

Earlier studies were mostly based on insects and they were quantitative analysis based on counting of cells and volumetric determinations of neuropil of brains. In 1979, Bieber and Fuldner noticed a remarkable enlargement of the mushroom body volume in the rove beetle *Aleochora* during the first twenty days after eclosion. The dramatic increase of the neuropil volume occurred by cell proliferation and not by cell growth because the number of cells (Kenion cells) increased of 71 %.

Cash and Carew (1989), by using the light microscope, distinguished neurons from glia cells by morphological features and they estimated the increase of the neuropile volume of the major central ganglia of *Aplysia,* a mollusk. They found a striking proliferation of central neurons occurring in each of the central ganglia simultaneously, during juvenile development.

Norlander and Edwards (1970), by using ^{3}H-thymidine, analyzed postembryonic brain development in the monarch butterfly *Danaus*. They showed that the initial small population of neurons in the newly-hatched larval brain increases rapidly by newly produced and differentiating neurons. They showed the presence of neuroblasts (asymmetrically dividing stem cells) whose activity, once begun, is continuous and without fluctuations corresponding to the molting cycle. Also in the larvae "antennal ganglion" three isolated neuroblasts were present. However, all these neuroblasts disappeared after pupation.

During the following years, it was always believed that the insect brain was unable to produce new neurons in adults because the neuroblasts which proliferate after embryogenesis disappeared after short time, before reaching the adult stage (Edwards 1969, Truman and Bate 1988). Earlier studies performed by Panov (1957, 1963, 1966. In: Carey et al., 1994), however, showed the presence of NBs dividing and producing ganglion mother cells in the mushroom bodies of immature stages of crickets. The limitation of these studies was the impossibility to follow the fate of the newborn neurons, though. Ito and Hotta (1992) carried out experiments in *Drosophila melanogaster*. Patches of neuroblasts were identified by BrdU after embryogenesis but the number of labeled cells decreased dramatically after the pupa stage, until they all disappeared. However, these results were disproved by a very recent result from Von Trotha and colleagues (2009) on cell proliferation in the adult fruit fly.

The complete sequence of events that characterize the second phase of neurogenesis (the first phase occurs during embryogenesis) in many species of insects was provided by Carey and colleagues in 1994 and 1996: they demonstrated neurogenesis throughout adult life of the cricket. In 1994, same authors reported the presence of undifferentiated cells, located dorsally in the mushroom bodies, of the adult house cricket *Acheta domesticus* that "persist, divide and give rise to interneurons". By the asymmetric division they could identify neuroblasts from ganglion mother cells, characterized by a symmetric cell division. This was the first study that showed that neurogenesis persists in adults of insects (Cayre et al. 1994, 1996). Two years later, the same authors showed the occurrence of neurogenesis in the mushroom bodies of more insects, belonging to various orthopteroid and coleopteran families (Cayre et al., 1996). However, not all insects' species show a second phase of neurogenesis in adulthood. Adult neurogenesis in holometabolous insects was so far only detected in species of Coleoptera (Bieber and Fuldner 1979, Cayre et al. 1996). In hemimetabolous insects, adult neurogenesis was found in Gryllidae (Cayre et al. 1994, 1996) and in the cockroach *Diploptera punctata* (Gu et al., 1999). It was not identified in Dictyoptera and Acrididae (Cayre et al., 1996) or in the honeybee (Fahrbach et al., 1995). In *Drosophila* it has been discovered recently (Von Trotha, 2009). Nordlander and Edwards (1970) could not show neurogenesis in the adult of the Lepidopteran species *D. plexippus* but Dufour and Gadenne (2006), by using BrdU, found newly born cells in the mushroom bodies of both adult males and females of the noctuid migrant moth *A. ipsilon*. Persistent NBs were shown to divide in early adult life to give rise to new neurons, also in 3-day-old adult insects.

So, to sum up, in some insect brains, adult neurogenesis only occurs among the Kenyon cells of the mushroom bodies, which are higher order multimodal integration centers receiving the input from the antennal lobes, the primary olfactory neuropils (Cayre et al. 1994, 1996, Gu et al. 1999, Dufour and Gadenne 2006). Adult neurogenesis in the mushroom bodies is lacking in several insect species and lasts only several days into adulthood (Gu et al., 1999).

Why? The biological significance of adult neurogenesis will be discussed later in this section.

5.1.3. Adult neurogenesis in Crustacea

The second major group of invertebrates in which adult neurogenesis has been studied is Crustacea, in particular decapod crustaceans. Contrary to insects, decapod crustaceans are characterized by a continuous growth in size trough repetitive molts and they increase the size up to 100.000-fold after having reached the adult shape (Schmidt, 1997).

In decapod crustaceans, adult neurogenesis occurs in two brain areas (Fig. 5.4): the optic lobes (Schmidt 1997, Sullivan and Beltz 2005a) and the olfactory system (Schmidt 2002, Beltz and Sandeman 2003, Sullivan et al. 2007a, b, Schmidt 2007a, b, Zhang et al. 2009).

5.1.3.1. The olfactory system in Crustacea

Beginning of the 20th century anatomical studies have identified the olfactory lobe (OL) as the olfactory processing area of decapod crustaceans because it receives sensory inputs from the 1st antenna (also called antennule). Bertil Hanström described the olfactory system as a "fibrillous substance (…) divided into two lobes (*Lo I* and *Lo II*, fig 1), which are connected at their side. (…) The cells lie mostly on the lower side of the lobus posterior (*Lo II*), but a smaller number of cells lie on the upper side of the lobus anterior (*Lo I*). (…) (Fig. 5.1A). The largest nerve-bundle in the brain of higher crustaceans connects the glomeruli of the deutocerebron with the glomeruli of the protocerebron (glomeruli pedunculares) (Hanström, 1925. Fig. 5.1A).

Almost 70 years later, Blaustein and collegues (1988) published the first detailed comparative morphological study of the brain of the spiny lobster and crayfish *Procambarus clarkii* (Fig. 5.1B). Extensive descriptions of the main structures of neural regions associated to the olfactory pathway of these two species were provided: the morphology and location of neuropiles, cell bodies, neuronal tracts and bundles of the brain with emphasis on the olfactory, accessory lobes and medulla terminalis. Olfactory lobes of the crayfish were described as spherical and made of glomeruli which occupy approximately 65% of the olfactory lobe and arranged radially (Fig. 5.1B). Interglomerular space, which is a matrix made of fine and large diamatere axons, vessels and glia cells, occupied the rest of the lobe (Blaustein et al., 1988). The volume of the accessory lobe was estimated to be three times that of the OL and to contain about 16,000 spherical glomeruli, smaller than those of the OL (Blaustein et al., 1988) (Fig. 5.1B).

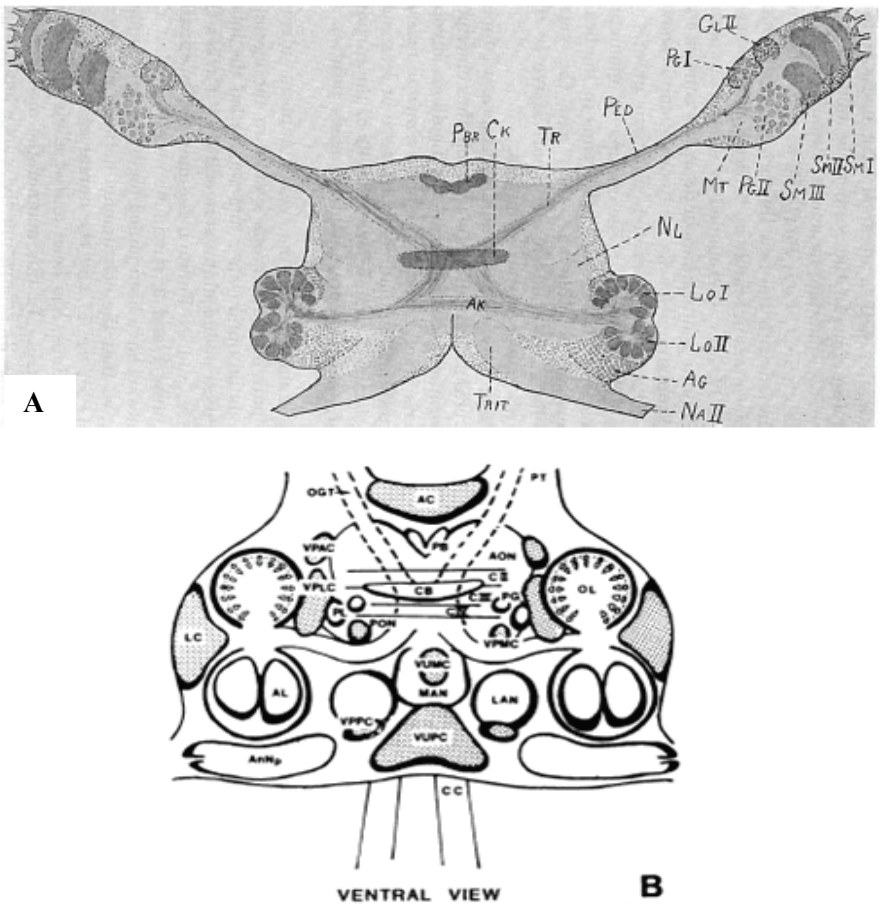


Fig. 5.1- A: Sketch of the brain of a decapod crustacean (*Carcinus maenas*) as described and draw by Hanström (1925). Abbr. are partialy reported in the text. B: reconstruction of the brain (ventral view) of a crayfish by Blaustein et al (1988). Abbr: AG, antennular glomerulus; AL, accessory lobe; AN, antennular nerve; AnN, antennal nerve; AnG, antennal glomerulus; AnNp, antennal neuropile; AON, anterior optic neuropile; CI, II, III, IV, optic commissures; CB, central body; CC, circumesophageal connective; LAN, lateral antennular neuropile; MAN, medial antennular neuropile; OGT, olfactory-globular tract; OL, olfactory lobe; PB, protocerebral bridge; PG, posterior optic neuropile glomeruli; PL, parolfactory lobe; PON, posterior optic neuropile; PT, protocerebral tract; TG, tritocerebral glomeruli; TN, tegumentary nerve; TNp, tegumentary neuropile (from Blaustein et al., 1988).

Few years later, Sandeman and colleagues (1992) presented a general morphological description of the brain of crayfish, crabs and spiny lobsters and introduced a common nomenclature for homologous structure to be used as a standard. Moreover, nerve roots, tracts and commisures were thoroughly described. Olfactory lobes receive the primary afferent endings of the chemioreceptors on antenna I. “The accessory lobes do not appear to receive a direct input from primary afferent axons, nor have motor efferents been found that have their synaptic fields there” (Sandeman et al., 1992). “The olfactory globular tract links the hemiellipsoid bodies and terminal medullae with the olfactory and

accessory lobes on both side of the brain by crossing the brain just dorsal to the central body neuropil. It typically contains very large numbers of small diameter axons" (Sandeman et al., 1992). The accessory lobes, adjacent to the olfactory lobes, are a unique feature of the decapod crustacean olfactory centers and they do not receive any primary afferent terminals but are involved in olfactory processing (Sandeman et al., 1995).

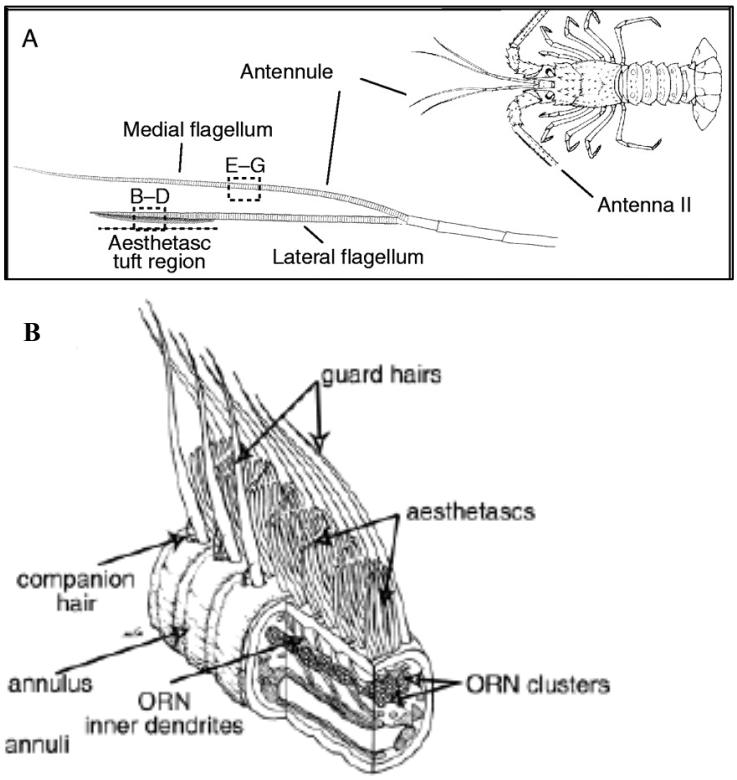


Fig. 5.2- A: Drawing of a spiny lobster showing antennae I (Antennula) and antennae II. The higher-magnification drawing of an antenna I shows the medial and lateral flagella. Aesthetascs are located exclusively on the distal half of the lateral flagellum, in the aesthetasc tuft region. Non-aesthetasc chemosensilla are located along the entire length of both the lateral and medial flagella. B: drawing of the antennulae (Antenna I) and the relative position of olfactory receptor neuron (ORN) clusters. Each aesthetasc is associated with approximately 300 ORNs (A from Steullet et al., 2002; B modified by Harrison et al., 2000).

Crayfish detect odors with their antenna I, located medially to the antenna II. Each antenna is composed of a lateral and a medial flagellum, each of which is segmented and contains up to 30 annuli (Tierney et al., 1986. Fig. 5.2A). In the distal segment of the lateral flagellum of the first antennae an arrangement of "aesthetasc", hair-like sensilla, is displayed (Tierney et al., 1986. Fig. 5.2B). Odors are detected by aesthetascs, which contain chemosensory receptor neurons (Hallberg et al., 1997). Adult aesthetascs contain between 40–110 olfactory receptor neurons (ORNs) and supporting cells and are organized along the adult antennule into two rows of 3–6 aesthetascs per annulus (Steullet et al., 2002. Fig. 5.2B).

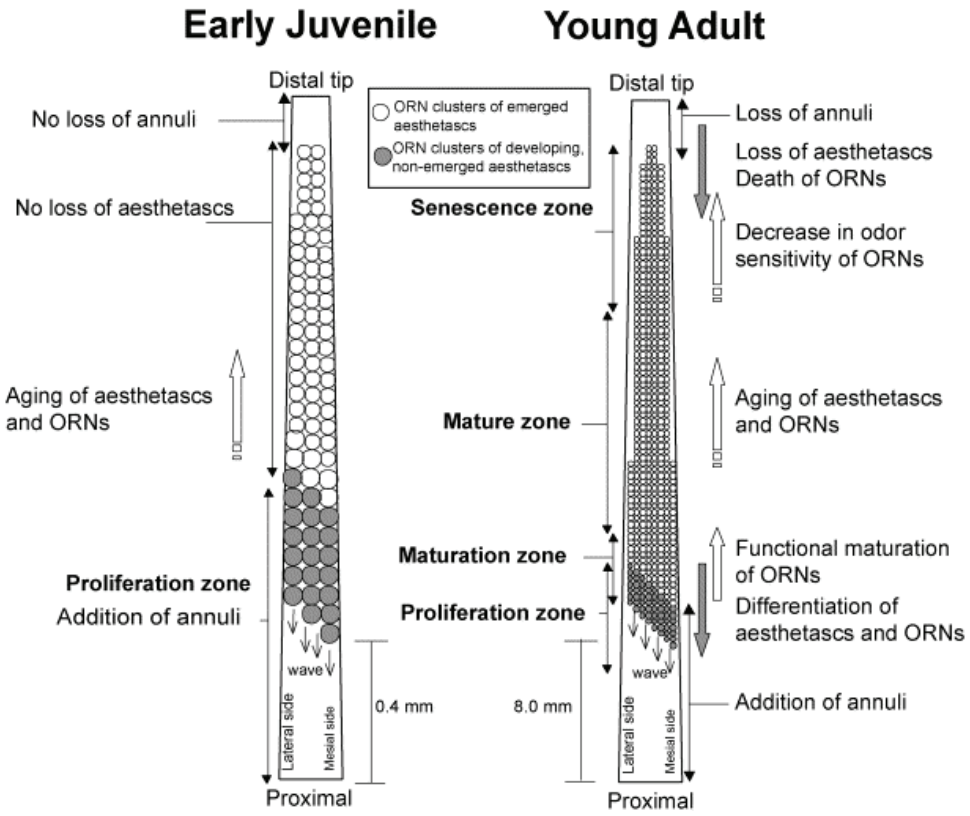


Fig. 5.3- Differences between early juvenile and adult spiny lobsters in the turnover of aesthetascs and their ORNs. Proliferation of new ORNs occurs in the early juvenile stage, the first benthic post-larval stage, and in adults. The proliferation is similar in early juveniles and adults when the increase is expressed as absolute numbers of added cells but much larger in early juveniles when expressed as a proportion of existing neurons (Derby et al., 2003). Death of the oldest neurons in the olfactory organ occurs only in adults and not in early juveniles (Derby et al., 2003).

The sensilla increase in number during body growth and at each molt they are continuously lost from the tip of the antenna I and replaced by new sensilla at the base of the array of the enlarging antenna, by a turn over of olfactory receptor neurons (ORNs) (Sandeman and Sandeman 1996, Steullet et al. 2002, Derby et al. 2003. Fig. 5.3). Derby and colleagues (2003) suggested that the addition of new cells in all life stages is an adaptation for indeterminate growth to replenish cells that die (Fig. 5.3). Associated with the molting, as the animal grows, is the addition of new sensory receptor neurons in the appendages (aesthetacs sensilla) (Mellon and Alones, 1993). It was thought that peripheral neurogenesis was a kind of must for the life-long neurogenesis detected in crustacean in the olfactory system until a study on the crab *Libinia emarginata* shed more light on the mechanism of neurogenesis (Sullivan and Beltz, 2005a). Unlike most decapod crustaceans, *Libinia emarginata* stops molting after reaching the complete

maturation. The study demonstrated that the proliferation and differentiation of olfactory interneurons in the brain continues even after the last molting suggesting "that peripheral neurogenesis is not a requirement for continuous neurogenesis in the central olfactory pathway" (Sullivan and Beltz, 2005a).

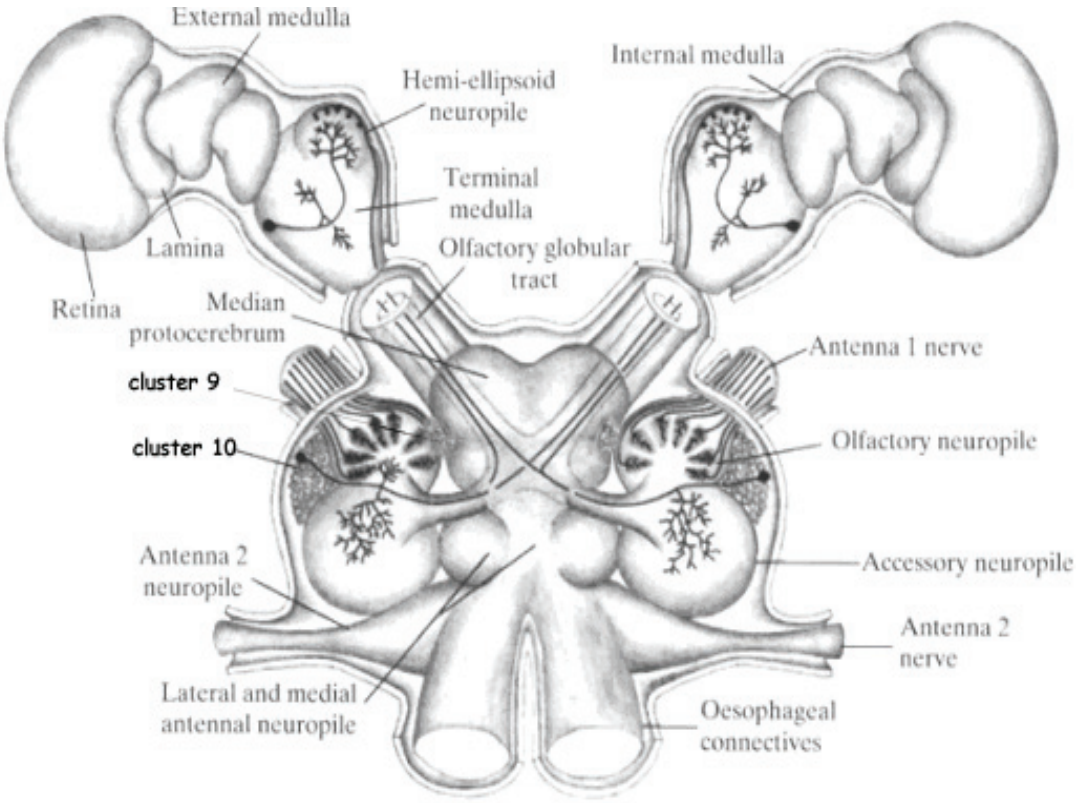


Fig. 5.4- Schematic diagram of the crayfish brain showing the olfactory pathway and the main associated structures of the first and second stage. Explanation of the first and second stage of the olfactory system are reported in the text (modified from Mellon et al., 1992a).

Olfactory neurons within the aesthetascs project their axons into the olfactory lobe which, together with the accessory lobe, represents the first stage of the olfactory pathway (Schmidt and Ache 1992, Schmidt 2007b. Fig. 5.4). The projections are unilateral and the olfactory lobes are not connected to one another (Sandeman et al., 1992). Glomeruli receive axons of olfactory receptor neurons expressing a specific odorant receptor. Within olfactory glomeruli, ORNs interact with local interneurons and projection neurons (Fig. 5.5). There are, in fact, different types of neurons which characterized the olfactory deutocerebrum. The cell bodies of local interneurons and projection neurons are located

outside the neuropils in separate cell clusters called cluster 9 (local interneurons) and cluster 10 (the projection neurons) (Sandeman et al., 1992. Fig. 5.4 and 5.5).

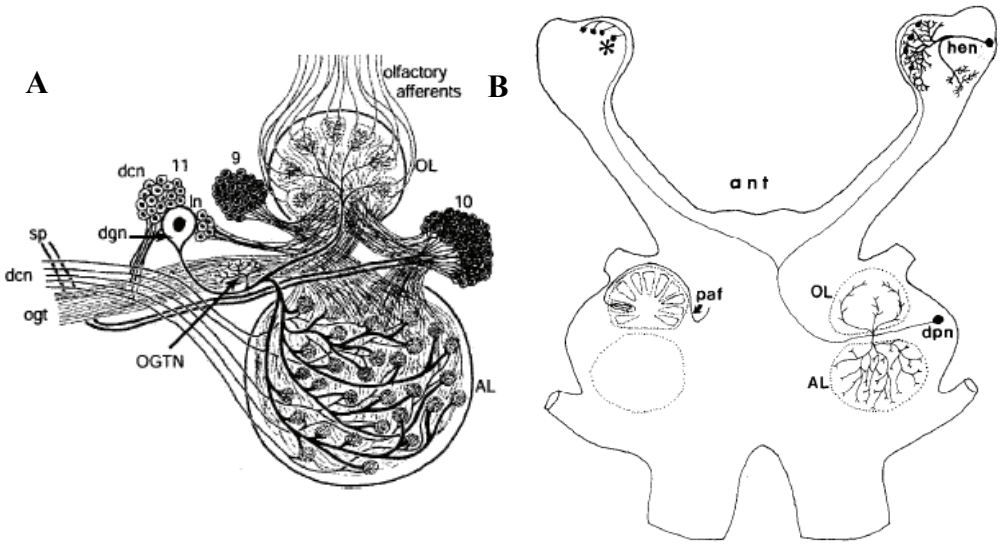


Fig. 5.5- **A:** Diagram of the olfactory pathway. OL: olfactory lobes; AL: accessory lobe; OGT: olfactory globular tract; cell body clusters 8, 9, 10, and 11. Olfactory afferent inputs project into the OL and enter the column-shaped glomeruli where they terminate in synapses with interneurons (paf in B). Local interneurons (cell bodies in 9) project into the OL and AL. Interneurons (cell bodies in 10) project into the OL or AL and have their axons in the olfactory globular tract (ogt). This tract runs from the OL and AL to the hemi-ellipsoid bodies and terminal medullae which are accomodated in the eyestalks (B). One group of local interneurons (dcn), whose somata are in 11, carry information from centers in the proto-, deuto- and trito-cerebrum to end exclusively in the glomeruli in the AL. A group of local interneurons (ln) in 11 terminates in the OL. The dorsal giant neuron (dgn) has its cell body in 11. Its neurite extends past a neuropile enclosed within the olfactory globular tract (OGTN) in which it branches to receive an input from the projection neurons exiting the lobes. **B**: dpn: deutocerebrum projection neuron; *: hemiellipsoid body; hen: hemiellipsoid interneuron; ant: anterior (A, B: from Sandeman and Sandeman, 2003. C: from Mellon et al, 1992a).

The local interneurons belong to several classes and they project exclusively to deutocerebral neuropils, OL and AL (Fig. 5.5), projection neurons either to the OL or AL, and the axons, joint with those of AL projection, project to the lateral protocerebrum. In fact axons, after leaving the OL and AL, bifurcate in the centre of the brain and project bilaterally to the higher regions of the brain, the second stage of the olfactory pathway, located in the eyestalk ganglia (Mellon et al., 1992a). The lateral protocerebrum neuropils are the hemiellipsoid body and the medulla terminalis which include different types of local interneurons whose somata is located in a cluster close to the hemiellipsoid body and called HBC (Mellon et al. 1992a, b, Schmidt 1997, Schmidt and Harzsch 1999) or soma cluster A (Sullivan and Beltz, 2005a) (Fig. 5.4 and 5.5).

5.1.3.2. Adult neurogenesis in the crustacean olfactory pathway

Although evidence for adult neurogenesis in the optic lobes so far has been obtained for only two species (*Carcinus maenas, Cherax destructor*) (Schmidt 1997, Sullivan and Beltz 2005b), evidence for adult neurogenesis in the olfactory pathway has been obtained for nine species of decapod crustaceans: the shrimp *Sicyonia brevirostris*, the spiny lobster *Panulirus argus*, the clawed lobster *Homarus americanus*, the crayfish C*herax destructor* and *Procambarus clarkii*, the hermit crab *Pagurus bernhardus* and true crabs *Carcinus maenas, Cancer pagurus, Libinia emarginata* (Schmidt 1997, 2001, 2007a, Sandeman et al. 1998, Schmidt and Harzsch 1999, Beltz et al. 2001, Hansen and Schmidt 2001, 2004, Song et al. 2005, Sullivan and Beltz 2005a, b, Sullivan et al. 2007a, b).

In 1994 and 1996, Harzsch and Dawirs were able to show, first in the ventral nerve cord and subsequently in the brain of spider crab *Hyas araneus* (Decapoda, Brachyura), post embryonic proliferative neuroblasts. In the ventral nerve cord, neuroblasts disappeared after the first metamorphosis but ganglion mother cells remained active until the juvenile stage (Harzsch and Dawirs, 1994). In the brain of the same species, the authors revealed the presence of mitotic activity until the second metamorphosis and a prolonged neurogenesis in the olfactory lobe (until crab 1 stage). At the same time, they registered a 10-fold increase of the olfactory lobe volume from hatching to the crab 1 (Harzsch and Dawirs, 1996a). The first studies on adult brain showed up the following years and they were based on counting neuronal somata or axons and by the mitotic marker BrdU. Adult neurogenesis was detected in the central olfactory pathway and hemi-ellipsoid bodies of the adult shore crab*, Carcinus maenas* (Schmidt, 1997) and crayfish (Sandeman et al., 1998) together with a turnover of peripheral olfactory receptor neurons in the antennulae (Sandeman and Sandeman, 1996).

In 1999, Harzsch and collegues traced neurogenesis from early embryonic stages through larval, juveniles and adult stages of the American lobster *Homarus americanus*. The authors could follow the long-term survival of the newly born cells by a pulse-chase experiment with BrdU (Harzsch et al., 1999b). In the same year, Harzsch and Schmidt confirmed the distribution of neurogenesis in the olfactory system of six more species of decapod crustaceans, by using BrdU (Schmidt and Harzsch, 1999). In 2003, an increase in the volume of the olfactory and accessory lobes and cell proliferation in cluster 9 and

10 of the crayfish was reported by Sandeman and Sandeman, who had also observed the influence of living conditions on the growth of the animals (Sandeman and Sandeman 2000, 2003).

In 2007, by pulse-chase experiment combined with neuron-specific antibodies Sullivan and colleagues could demonstrate the maturation and differentiation of newly-generated cells into neurons in cluster 9 and cluster 10 of the crayfish *Procambarus clarkii*. Authors also provided evidence on the nature of the precursor cells responsible for the adult neurogenesis. Glia cells were shown to participate to the production of newly-born cells and to reside in a specific area of the brain, on the ventral surface of the brain, called ***neurogenic niche*** (Sullivan et al., 2007a).

Following studies analyzed and hypothesized the role of this structure, the type of cells and mechanisms involved, and the distribution of this peculiar apparatus in other species (Sullivan et al. 2007a, b, Schmidt 2007a, b, Song et al. 2009, Zhang et al. 2009).

5.1.4. The stem cell niche: function and development

A stem cell niche is "a specific anatomic location", "it constitutes a basic unit of tissue physiology, integrating signals that mediate the balanced response of stem cells to the needs of organisms" (Scadden, 2006). "The simple location of stem cells is not sufficient to define a niche. The niche must have both anatomic and functional dimensions" (Scadden, 2006). The role of stem cells is fundamental but lately the idea is that the specialized microenvironment surrounding the stem cells is crucial: the "niche hypothesis" (Schofield, 1978).

The first niche to be defined and in fact the best studied is the niche of the *Drosophila* ovary (Xie and Spradling, 2000. Fig. 5.6A). The ovary is composed of a series of tubes (ovarioles) producing eggs. Each of them shows, at the anterior tip, a structure called germinarium, each of them hosting two or three GSCs (germ stem cells). GSCs cells are surrounded by specialized somatic cells called cap cells. Terminal filament cells and inner sheath cells are also part of the population of the niche. GSCs can be distinguished by the size, location and cytoplasmatic organelle (fusome) on the side of the GSC which is in contact with the cap cells (de Cuevas and Spradling, 1998. Fig. 5.6A). Xie and Spradling (2000) found that the niche itself was responsible for providing signals that

control stem cell self-renewal by repressing expression of specific genes in the stem cells. In this scenario, not stem cells but the relationship between stem cells and their niche appears fundamental for the whole process of generating new cells. Apart from intrinsic genetic programs within the stem cells, most of the regulation in fact comes from the cells which make up the niche. They are in charge for self-renewal, proliferation, differentiation of stem cells. Moreover, in the Drosophila ovary the niche is even able to help to reverse differentiated cells back into GSCs. BMP signaling can reverse differentiated germ cell cysts back into functional GSCs (Xie and Spradling, 2000). In this way, stem cells lost by normal or induced differentiation are replaced within their niches. Even more fascinating is the capacity of the niche to work independently of resident stem cells and to be able to reprogram newly introduced cells to become stem cells (Xie and Spradling, 2000). It has also been demonstrated that DE-cadherin accumulates between cap cells (surrounding cells which form the niche) and GSCs inside the niche and is essential for anchoring stem cells in their niche (Song et al., 2002). Consequently, in the *Drosophila* ovary, the characteristic structure of the niche provides a sufficient internal surface area which gets in contact with two or maximum three stem cells (Fig. 5.6A). A special cell-cell junction by DE-cadherin is between stem cells and niche cells which hold the stem cells and prevents them from moving away and differentiate (Song et al., 2002). The combined action of specific genes expressed by the cap cells regulates these tight contacts (Fig. 5.6B). When one of the daughter cell loses the contact with the cap cells, it moves away and differentiates. At the same time, the neighboring GSC divides and the daughter cell occupy the space vacated by the departed stem cell and becomes the new stem cell (Xie and Spradling, 2000). Song and colleagues (2002) proposed also a model for recruiting stem cells and anchored them in the niche. The expression of DE-cadherin in primordial germ cells is essential to be recruited by the niche and to maintain their identity. The niche cells are, hence, crucial for the regulation of stem cells and authors suggest that stem cells in different tissues and different organisms may be regulated by using the same strategy. In the *Drosophila* testis, five to seven stem cells are anchored on differentiated somatic hub cells, in *Caenorhabditis elegans* distal tip cells have been directly implicated in the maintenance of the stem cell population, in *Arabidopsis* meristem an organizing center located nearby is required to

maintain meristem stem cells, the plasticity of some mammalian stem cells depends on niches that can reprogram stem cell identity (Xie and Spradling, 2000).

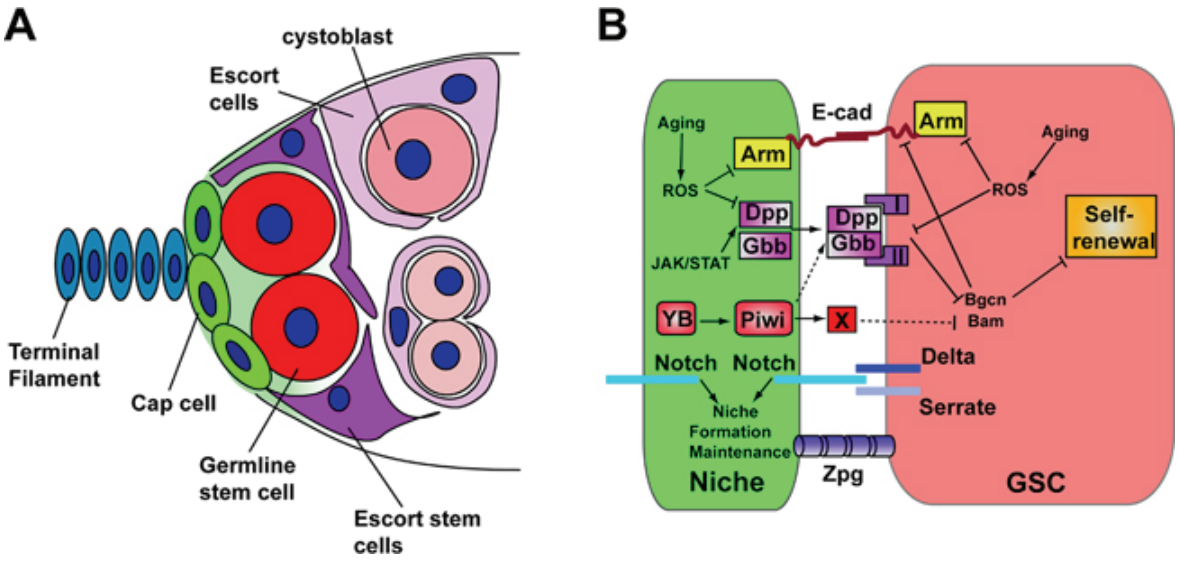


Fig. 5.6- Structure and signaling mechanisms of the *Drosophila* ovarian GSC niche. **(A)** Cap cells (green) and escort stem cells (purple) function as a niche to maintain GSCs (red, green shade representing the niche influence), allowing germ cells (pink) outside the niche to differentiate. **(B).** Notch signaling controls the niche formation and maintenance, while aging affects BMP signaling activity and E-cadherin expression in niche cells. The Dpp/Gbb-mediated BMP signaling pathway and the Yb/Piwi-mediated unknown signaling pathway together repress expression of differentiation-promoting genes including *bam*, thereby maintaining GSC self-renewal. Intrinsic GSC aging affects E-cadherin expression and BMP reception. E-cadherin-mediated cell adhesion is required for GSC niche anchorage and competition for niche occupancy, while Zpg-containing gap junctions are required for GSC survival. (Text and figure from Xie and Spradling, 2000).

The niche in the germarium of the *Drosophila* ovary has also been one of the first whose development has been partly analyzed. GSCs cells may derive from pole cells, a group of cells initially located at the posterior pole of the blastoderm embryo. During embryogenesis they migrate until they end up where gonads will appear and form the primitive gonads. During embryogenesis and larval stages, these cells proliferate to make 80-100 germline cells (these cells will give rise only to a single differentiated cell type, the gamete) per ovary. And the end of larval development, pole cells stop to proliferate. Gonzalez-Reyes (2003) traced back the appearance of the niche to the end of the larval stages, when somatic cells of the gonad proliferate, differentiate and subdivide into 70-20 ovarioles. At this stage, terminal filaments cells and cap cells form the anterior part of the

germarium (Fig. 5.7). The niche is thus initially formed in the absence of GSCs. Close to cap cells are two or three germline cells (that might be precursors of adult GSCs) that derive from pole cells. The hypothesis suggested by Gonzalez-Reyes (2003) is that during the larval-pupal transition, differentiation of cap and filament cells occurs.

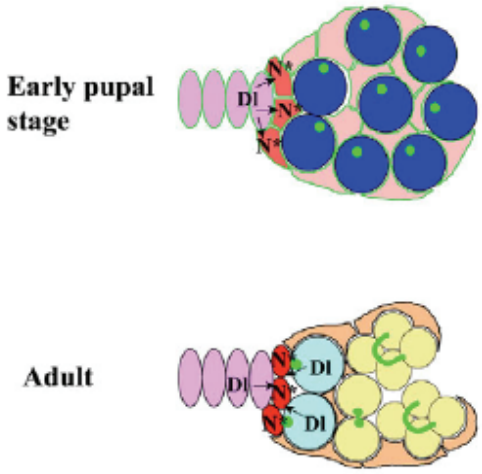


Fig. 5.7- Development of the niche of the *Drosophila* ovary. Terminal filament cells (in pink) express DI protein which activates Notch signaling in their adjacent somatic cells (red-orange) to induce cap formation. Once the cap cells are formed, they get in contact with germline cells (blue) which in turn become GSCs (light blue). Cells which are not in contact with cap cells will become escort cells or inner sheath cells (yellow and brownish) (from Song et al., 2007).

Once terminal and cap cells are organized into a niche, they induce the germline cells in contact with cap cells to adopt a cell fate (Fig. 5.7, Adult stage). The idea is that "the apposition of cap cells and pole cells pushes the latter into acquiring a stem cell fate". Afterwards, the new GSC initiates to divide asymmetrically (Gonzalez-Reyes, 2003). So, apparently stem cells are recruited among the pool of germline cells proliferated by pole cells during larval stages. A compulsory requirement seems to be the establishment of cadherin-mediated junctions to tight germline cells to cap cells.

Some years later, Song and colleagues (2007) added some further important discoveries on the niche formation and recruitment of GSCs in the *Drosophila* ovary niche. *Notch* signaling controls the formation of the GSC niche and cap cells determine the size of the niche (Fig. 5.7).

More explanations of the model are provided in Fig. 5.7.

5.1.5. The Neurogenic Niche in Crustracea

Neurogenic compartments have been identified in many species of vertebrates and invertebrates (Tab. 5.1).

Taxon	Species	Site	References
Cnidaria	*Hydra viridissima*	Gastric region	Sakaguchi et al. (1996)
Insecta	*Acheta domesticus*	MB	Cayre et al., 1994; Cayre et al., 1996; Scotto-Lomassese et al., 2000, 2002, 2003
	Gryllomorpha dalmatina	MB	Cayre et al. (1996)
	Gryllus bimaculatus	MB	Cayre et al. (1996)
	Tenebrio molitor	MB	Cayre et al. (1996)
	Zophobas sp.	MB	Cayre et al. (1996)
	Harmonia axyridis	MB	Cayre et al., 1996
	Oncopeltus fasciatus	MB	Cayre et al. (1996)
	Alaochara curtula	MB	Bieber and Fuldner (1979)
	Stagmomantis carolina	MB	As reviewed by Cayre et al. (2002)
	Agrotis ipsilon	MB	Dufour and Gadenne (2006)
	Drosophila m.	AL	Von Trotha et al, 2009
Crustacea	*Carcinus maenas*	LC, HBC	Schmidt (1997) and Hansen and Schmidt, 2001 and Hansen and Schmidt, 2004
	Libinia emarginata	LC, MC, HBC	Sullivan and Beltz (2005)
	Pagurus bernhardus	LC	Schmidt and Harzsch (1999)
	Cancer pagurus	LC	Schmidt and Harzsch (1999)
	Hyas araneus	LC	Harzsch and Dawirs (1996)
	Panulirus argus	LC, MC	Schmidt and Harzsch (1999) and Schmidt (2001)
	Homarus americanus	LC	Harzsch et al. (1999) and Schmidt and Harzsch (1999)
	Cherax destructor	LC	Schmidt and Harzsch (1999) and Sandeman and Sandeman (2000)
	Sicyonia brevirostris	LC	Schmidt and Harzsch (1999)
	Procambarus clarkii	OL	Sullivan et al. (2007)
	Procambarus sp. (Marbled crayfish)	OL	Sintoni et al (in prep)
Molluscs	*Aplysia californicus*	Unknown	Cash and Carew (1989)
Urochordates	*Ciona intestinalis*	Dorsal plexus	Mackie (1995)

Tab 5.1- Adult neurogenesis compartments in invertebrates The present study on the Marbled crayfish is included (red circle). Abbr: AL, accessory lobe; HBC, hemiellipsoid bodies; LC, lateral soma cluster; MB, mushroom bodies; MC, medial soma cluster; Ol, olfactory lobe (modified from Lindsey and Tropepe, 2006).

Proliferation of cells is usually detected by BrdU, but the presence of a mitotic marker does not imply that such cells were generated *in situ*. One example of migration of cells from the place of origin to the final destination is given by the crayfish *Procambarus clarkii*, in which a particular structure called the *neurogenic niche*, responsible for the production of new cells, was found on the ventral surface of the brain of adult crayfish (Sullivan et al., 2007a. Fig. 5.8). The occurrence of neurogenesis after embryogenesis implied in fact that neural stem cells, able to divide to multiplicate themselves but also to

produce progenitor cells which in turn give rise to different cell types, were present also in the adult brain. The questions to be answered were, thus, where such cells are located and what type of identity they have (Sullivan et al., 2007a). In the brain of adult *Procambarus clarkii*, two zones which show proliferation of cells are localized near cluster 10 and 9, the LPZ (lateral proliferative zone) and MPZ (medial proliferative zone), are connected to a specialized structure, the niche, placed in between, and linked to them by two filamentous streams. The streams are made by processes of cells whose cell bodies are housed in the niche itself and whose identity has been suggested to be glial (Sullivan et al., 2007a. Fig. 5.8).

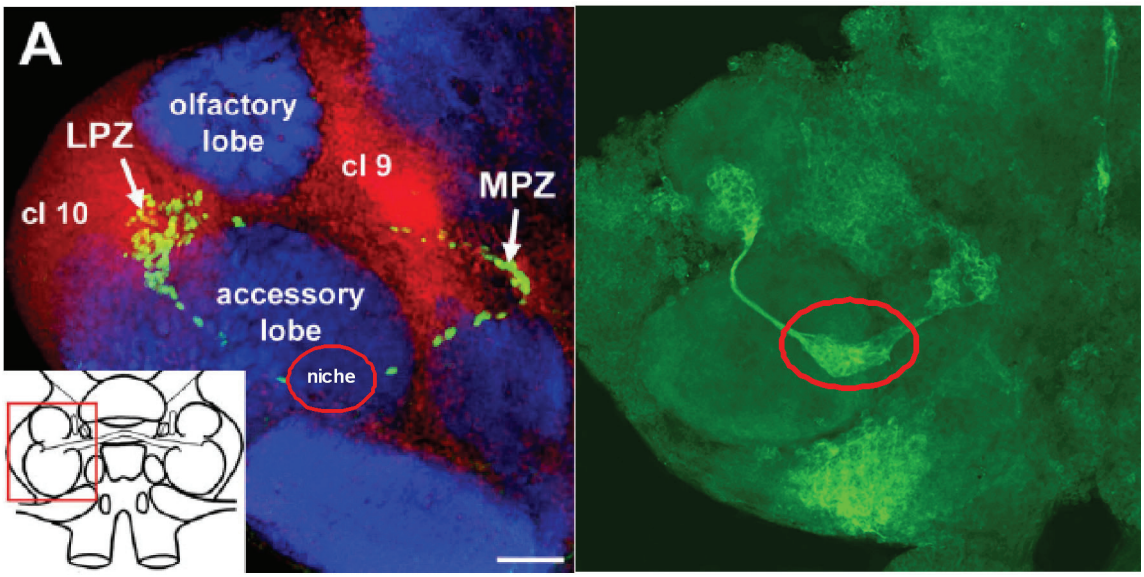


Fig. 5.8- Schematic diagram of the left side of the crayfish brain showing the olfactory pathway and the main associated structures in the deutocerebrum. A: BrdU positive cells (green) are found in the LPZ (lateral proliferative zone) near cluster 10 and MPZ (medial proliferative zone) near cluster 9. The two zones are connected by a stream of labeled cells which takes origin from a structure in the middle, circled in red, and visible in B: the niche. The niche is labeled with gluthamine synthetase, a glia cell marker (in green, B). A: modified from Sullivan et al., 2007a; B: images from Sintoni S).

According to the definition of Morrison and Spradling (2008), a niche is a "specialized local microenvironment where stem cells reside and that directly promote the maintenance of stem cells".

In *Procambarus clarkii*, the niche is localized on the ventral surface of the brain. It is an oval structure and in the middle an unlabelled region is visible and still to be characterized. Sullivan and colleagues (2007a) found that the central structure is positive to DN-cadherin antibody which detects this cell adhesion molecule expressed in some

vertebrate basal laminae. The niche cells are reactive to gluthamine synthetase, a marker which has been seen to stain glia cells in the brain of crayfish, spiny lobster *Panulirus argus* and crabs (Linser et al. 1997, Allodi et al. 2006). The processes of the niche cells fasciculate into tracts to form the two streams which end up in the proliferative zones. Intracellular staining of cells with Lucifer yellow showed that these cells are bipolar. Exposure to BrdU resulted in some labeled cells which, in turn, by using a M-phase marker (phospohistone H3) indicated that they also undergo mitosis. By using another cell cycle marker (G1 phase marker, MCM2-7), other labeled cells were highlighted in the niche. Together these results provided evidence for mitotically active cells (Sullivan et al., 2007a). In incubation experiments with BrdU, cells in the streams were also labelled and by double nucleoside analogue-labeling experiments it was demonstrated that the streams are migratory tracts, since cells move from the niche towards the proliferative zones. The experiment was performed by using two nucleoside analogues, BrdU and IdU (iododeoxyuridine), which both get incorporated into DNA during the S-phase of the cell cycle but they can be detected by two different antibodies. In this way, it is possible to detect cells in S-phase that were exposed to one nucleoside days before the second incubation with the second nucleoside analogue (Sullivan et al., 2007a).

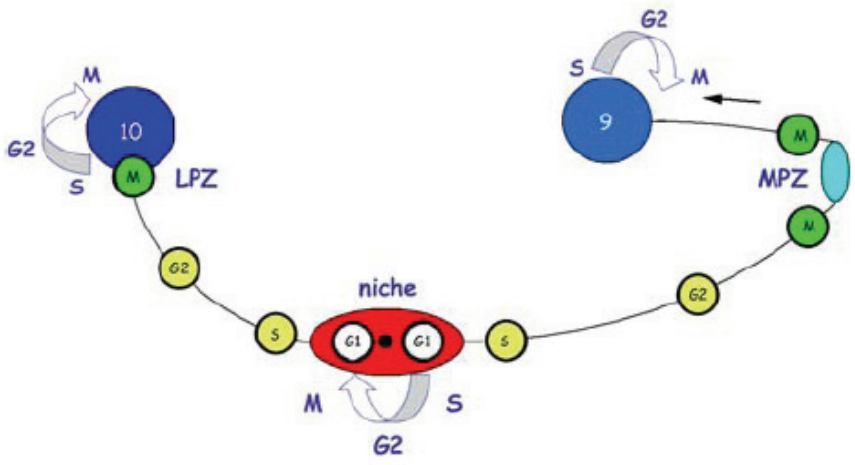


Fig. 5.9- Schematic diagram of the hypothetical mechanism of the crayfish niche. Quiescent cells with glia features reside in the niche. Here, they divide asymmetrically, resting in G1 phase. Once they divide, they produce one self-renewed precursor cell and one daughter cell. Daughter cells migrate to the LPZ and MPZ (proliferative zones) progressing from S-phase to G2 phase. Close to the PZs, cells undergo mitosis and by more divisions generate immature neurons in cluster 9 and 10. Most of these immature cells will differentiate into olfactory interneurons (Nomenclature and text are from Sullivan et al., 2007a).

This type of experiment gives evidence for the dynamic of cells and the direction of movements can be thus analyzed. The work of Sullivan and colleagues (2007a) together with more investigations (Sullivan et al., 2007b) performed by using specific cell cycle markers, different incubations time and pulse-chase experiments with BrdU, ended up in a model which summarizes the function and mechanism of the niche and its cells (Fig. 5.9).

In the same year, totally in contrast with the previously explained findings of Sullivan et al (2007a), Schmidt published his results on the spiny lobster, *Panulirus argus*, arguing that responsible for neurogenesis in late juveniles and adults are still neuroblasts, the asymmetrically dividing stem cells involved in neurogenesis during the embryo life. By multiple injections of BrdU over a period of 2 days, one large "putative neuroblast" was found inside (and not outside) each soma cluster of the deutocerebrum (Schmidt, 2007a. Fig. 5.10).

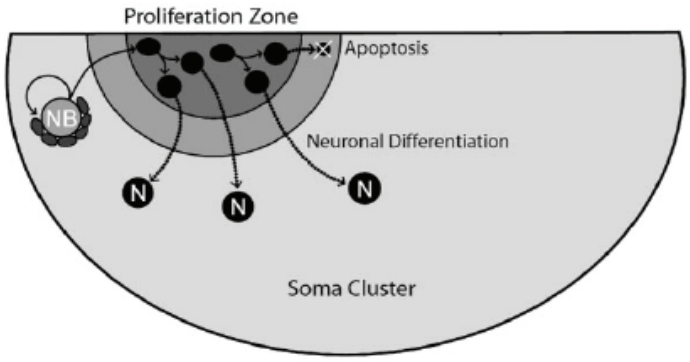


Fig. 5.10- Schematic diagram of the hypothetical mechanism of the neurogenesis in the adult spiny lobster proposed by Schimdt (2007a). One large putative neuroblast (NB) close to the proliferation zones funtions as neuronal stem cell. By rapid asymmetric divisions, it divides into one self-renewed precursor cell and one daughter cell. Daughter cells migrate to the proliferative zone and there divide symmetrically. After the mitosis, daughter cells are pushed out the proliferation zone and either differentiate into neurons or die by apoptosis (Schmidt, 2007a).

By using the same glial marker, gluthamine synthetase, the author could not obtain distinct labeling. The conclusion of his study was that adult neurogenesis mechanism resembles the one occurring during embryogenesis, in which neuroblasts divide asymmetrically and rapidly to produce one self-renewed precursor cell and one ganglion mother cell. Ganglion mother cells, in turn, divide asymmetrically to produce two

neurons (Schmidt, 2007a). The only difference is that the putative adult neuroblast is associated with specialized group of cells that somehow give support and keep alive this special neuroblast, characterized by an unusual lifelong capacity of proliferation (Schmidt, 2007a. Fig. 5.10). This clump of cells, surrounded by fibrous material, was considered the stem cell niche in the spiny lobster (Schmidt, 2007a).

In 2009, Schmidt and colleagues published results on adult neurogenesis in *Procambarus clarkii*, too (Song et al., 2009). Although in partial accord with Sullivan and colleagues concerning the presence of a specific structure that they call "*neurogenic complex*", the hypothesis of involvement of neuroblasts instead of glia cells as stem cells was still proposed. The study was done by using BrdU (multiple injections over a short time) and by morphometric analysis of the size and shape of the BrdU nuclei of the niche cells. The conclusion of the study was that a large neuroblast is present in each subdivision of the clump of cells and, thus, each of them contributes to each proliferative zone. Contrary to the quiescent cells in the glia niche, here the neuroblast divides rapidly to replenish the neuronal precursors that move to the proliferative zones (Song et al., 2009).

5.1.6. Comparing neurogenesis in Vertebrates

The controversy about the identity of the stem cells and the modality by which they reproduce themselves is made further complicated by comparisons with other groups or species. For instance, glia cells dividing slowly and the progeny migrating along a glial path resembles the scenario seen in mammals (Doetsch, 2003a). In mammals, the subventricular zone (SVZ) is a layer of dividing cells, neural stem cells (NSCs), which share common features with astrocytes, such as morphological characteristics and expression of glial protein (Ma et al., 2009). These cells give rise to the so called "neuroblasts" that migrate along the rostral migratory stream (made of processes of the slowly dividing astrocytes) (Curtis et al., 2007) towards the olfactory bulb where they differentiate into specific classes of neurons (Doetsch and Scharff 2001, Doetsch 2003b. Fig. 5.11). In order to avoid misunderstandings in terms of nomenclature, in vertebrates astrocytes are the stem cells that, by dividing, generate immature migrating neuroblasts ("blasts" indicates they are still dividing. Doetsch, 2003b) that, contrary to those in invertebrates, are post-mitotic cells, not self-renew cells (precursor cells).

Further features of the mammal niche are the presence of extracellular matrix and basal lamina, together found in the crayfish by Sullivan and colleagues (2007a).

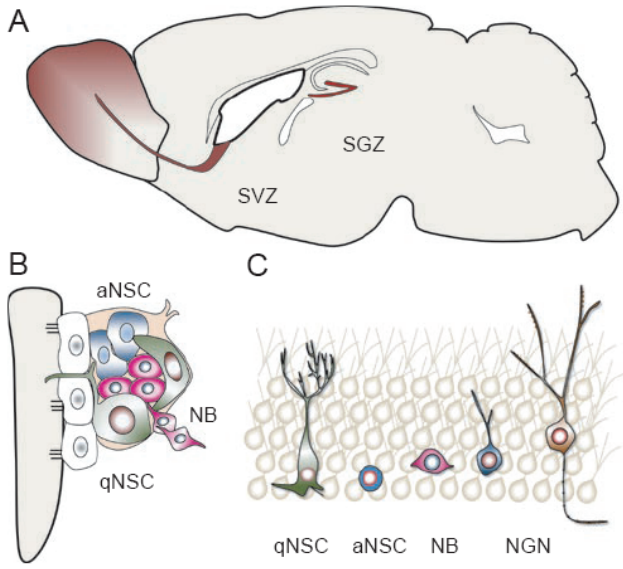


Fig. 5.11- Schematic drawing of the adult mammalian brain in mice. A: Adult neural stem cells (NSCs) are present in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus. B: Adult NSCs in the SVZ. Quiescent adult NSCs (qNSC), which share features with astrocytes and ependymal cells, have the cell bodies in the SVZ and the apical surfaces in contact with the ventricle. Active NSCs self-renewing adult (aNSC) are located in the SVZ and they produce neuroblasts (NB) that migrate towards the olfactory bulb. C: Adult NSCs in the SGZ. Quiescent or dormant adult NSCs correspond to radial glia-like cells, some of which transit to actively self-renewing adult SGZ NSCs and give rise to neuroblasts (NB) and newly generated neurons (NGN) (Ma et al., 2009).

In mammals, astroglia, ependymal cells, vascular cells, NSC progeny and neurons constitute the major population of the neurogenic niche. Latest hypothesis are about the fundamental role of astroglia in regulating almost every developmental processes of adult neurogenesis (Song et al. 2002, Barkho et al. 2006) as well as interferences of vascular cells that, by making permeable the blood vessels, allows the entrance of specific signals to regulate adult neurogenesis (Ma et al., 2009). At the time of the paper of Song and colleagues (2009), it was believed that neuroblasts were still present after embryogenesis and responsible for the "late" neurogenesis in the mushroom bodies of some insects. Insect neuroblasts, in fact, remain active for some days, even weeks into adulthood, but they eventually die by apoptosis (Gu et al., 1999). By comparing the situation in insects, Song and colleagues (2009) but also Schmidt (2007a, b) argued that, because crayfish and lobster are closely related and they both are related to insects, it must be likely that

the adult neurogenesis shares similar conditions and characteristics. As logic consequence, the presence of neuroblasts after embryogenesis was understandable.
In *Drosophila*, adult stem cells have recently been discovered in the adult gut (Ohlstein and Spradling 2006, Micchelli and Perrimon 2006) and in the malphigian tubules (Singh et al., 2007) but the brain of the adult fly was considered not subject to cell proliferation, not even in the mushroom bodies, because neuroblasts stop dividing before eclosion (Ito and Hotta, 1992). A recent paper published after Song's article, reports data that demonstrated that cell proliferation takes place in the antennal lobes of the adult Drosophila brain and, even more unanticipated, most of the dividing cells (about 86%) are positive to Repo, a protein expressed by glia (Von Trotha et al., 2009).
So, to conclude, the debate is still open and surely more experiments are needed to clarify the real context in which adult neurogenesis occurs in Crustacea and, in general, in Invertebrates.

5.1.7. Looking for the meaning of adult neurogenesis

The big question that needs to be answered is about the functional significance of adult neurogenesis, the role of newborn neurons and why the brain requires such complex structures like the niche. "Expedients" like synaptogenesis or new arborizations are also at the basis of brain plasticity but, in the case of a second phase of neurogenesis, production of new neurons may represent the only mean to achieve specific goals in the adult brain, by adding new competences or abilities to the circuit. The phenomenon appears to be widespread, especially among vertebrates and a biological meaning must be on top of it. On the other hand, explanations should be given also to the unusual distribution of adult neurogenesis among some insect species: in some of them stem cells are present, in others not. Behavior was thought to be the answer to this issue but complex behavioral aspects showed by bees and ants, for instance, are not justified by the post embryonic neurogenesis, since these animals do not show it at all. Conversely, an increase of synaptic contact has been showed to occur in the mushroom bodies and this event may justify the brain plasticity (Whiters et al., 1993). In the house cricket, *A. domesticus*, adult neurogenesis persists throughout the insect life and it was calculated that adult-born neurons represent about 20% of the total cortex volume and it is not

accompanied by cell death, but cells increased in number and they increased also the cell density (Malaterre et al. 2002, Carey et al. 2007). This suggests that it must have a crucial involvement in some primary function. Moreover, it has been demonstrated that many factors influence the rate of neurogenesis, such as hormones, neurotransmitters, growth factors, social relations, environment conditions (Cayre et al., 2002). Cayre and colleagues (2005) suggested that new neurons may be responsible for the oviposition behavior of females and that the timing of their appearance and differentiation may control the timing of female egg-laying behavior.

Scotto-Lomassese and colleagues (2003) showed that adult neurogenesis in insects was involved in olfactory learning and memory: repression of adult neurogenesis using irradiation (ionizing rays), which suppressed NBs and progeny, prejudiced the processing of olfactory information and animals exhibited weak learning when olfactory cues alone were used. In the moth, persistent neurogenesis in the adult mushroom bodies is responsible for the olfactory plasticity by increasing sensitivity to pheromones (Dufour and Gadenne, 2006).

Experiments of serotonin depletion in crustaceans showed that serotonin stimulates the proliferation of progenitor cells (Benton and Beltz, 2001).

In Crustacea, due to the animal life span of years or even decades, a continuous production in one of the most important system of the animal (olfactory) needs a specific structure as a support for maintaining the turnover of neurons necessary for the adaptation and survival of the animal in the changing environment. In particular, the hypothesis is that new projection neurons are needed for detecting new odor stimuli. Projection neurons usually project into glomeruli which do not increase in number and this means that new projection neurons afferents simply get accommodated into the pre-existing glomeruli (Helluy et al. 1996, Schmidt 2007b). This background could sustain the hypothesis that what changes in adult life in consequence of newborn neurons is the odor sensitivity, for instance (Schmidt, 2007b). The reason why in some insects such event is not widespread could lie in the short life expectancy of most of the insect species (weeks, sometime months). In adult crustacean it has been proved that months are required to neurons to mature, in insects such long process of maturation would be impossible (Sullivan and Beltz 2005b, Sullivan et al. 2007a).

In vertebrates, experiments with songbirds and rats showed how the new production and increase in number of neurons improve learning abilities and task (Scharff et al. 2000) and so neurons are functionally integrated into previous circuits.

Doetsch and Hen (2005) showed that in rodents immature neurons have distinct electrophysiological properties, different from the old neurons, suggesting that the continuous addition of neurons help adapting the animal to changing environments. Behavioral changes have been also seen to depend on new neurons added in the hippocampus: adult neurogenesis is necessary for antidepressant action (Santarelli et al., 2003). Surely more investigations are needed to shed light on such widespread and biologically crucial phenomenon occurring in the brain of many adult species.

5.1.8. More issues as premises to the niche project

One appealing question is why a closed system like a niche has been chosen for driving the adult neurogenesis process. According to Morrison and Spradling (2008), niches have to ensure that stem cells remain in the niche after stem cell division. In fact, usually one daughter cell remains in the niche and one exits and differentiates, thus stem cell niches must prevent that external cells entry and displace the resident stem cells. “The need to discharge daughters without admitting competitor cells may have contributed to the evolution of niche structure as well as self-/nonself-recognition by the immune system” (Laird et al., 2005). Actually, as reported by Margolis and Spradling (1995), some invertebrate stem cells are displaced from their niche on a regular base and even some mammalian stem cell types face the same fate. Probably the reason for such replacement could be the attempt to eliminate mutations which have accumulated in the original stem cell (Jin et al., 2008). Spradling and colleagues (2001) suggest that stem cells might have first appeared when niche acquired the capacity to sequester and control undifferentiated embryonic cells. In general, it can be said that the primary function of the niche is to anchor stem cells, as well as to provide inputs and signals to stem cells.

Regarding the niche cells, a matter to think about is their identity. It is in fact necessary to clearly distinguish between stem cells, progenitor cells, precursor cells and neuroblasts. Unfortunately many definitions are available in different articles and often stem and progenitor cells are classified based on either *in vivo* or *in vitro* functional differences.

Seabergand and van der Kooy (2003) referred to a "disturbing trend in stem cell biology" the abandonment of rigorous definitions of stem and progenitor cells in favor of more ambiguous, all-encompassing concepts". In fact, according to the type of tissues in which stem or progenitor cells are found, a broader re-definition is given and in this way the designation of the real stem cell come into discussion again (Seabergand and van der Kooy, 2003). Let s say that debates on the real meaning of the "evolving "concept of stem cells are beyond the scope of the thesis. Moreover, in the final discussion, some more thoughts on stem/progenitor/precursor cells definition will be provided in order to argument results and hypothesis. Besides, a scheme with the main characteristics is reported:

Characteristic	Neural stem cell	Progenitor cell
Self-renewal *in vivo*	Unlimited: for the lifetime of the organism	Limited: transient
Self-renewal *in vitro*	Unlimited: can reach maximum number of population doublings before transformation	Does not reach maximum number of population doublings
Potentiality	Multipotent (able to generate neurons, astrocytes and oligodendrocytes)	Most often unipotent: can be multipotent
Maintenance of self-ren. and multipotentiality	Yes	No

Modified by Seabergand and van der Kooy (2003)

In arthropods, the stem cell mode has been already described in Chapter 2 (see also Fig. 5.12). In myriapods and chelicerates, neurons are generated by immigration of post-mitotic neural precursor cells (np), in insects and crustaceans specialized cells, neuroblasts (NBs), divide in a stem cell-like manner (the stem cell mode) (see review: Stollewerk and Simpson, 2005) (Fig. 5.12). In insects, they bud off smaller ganglion mother cells (GMCs) which in turn divide once to generate ganglion cells (GCs) that differentiate into neurons and glia cells (Bate 1976, Hartenstein et al. 1987). Similar division pattern may occur in higher crustaceans, but direct evidence is still missing, although examples for such division have been shown by Harzsch and Dawirs (1994, 1996a), Harzsch et al. (1998), Ungerer and Scholtz (2008) (Fig. 5.12).

In mammals, NSCs represent a special cell type that has the capacity to self-renew and generate different neural lineages. The identity of adult NSCs, which are believed to be largely quiescent *in vivo*, is although under debate. As mentioned above, these cells

expresse glial fibrillary acidic protein (GFAP) and exhibit some morphological radial glial properties and thus are thought to be special astrocytes. Apparently, in fact, NSCs are able to generate neurons, astrocytes, and oligodendrocytes in the adult brain of mammals (Duan et al., 2008).

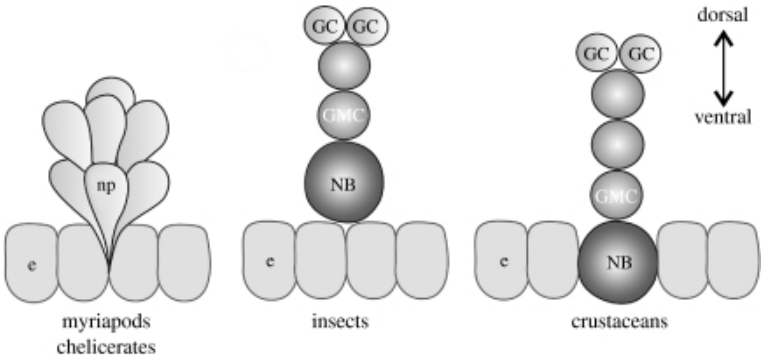


Fig. 5.12- Neurogenesis in arthropods. Explanations are reported in the text (from Ungerer and Scholtz, 2008).

As said above, in Crustacea a defined pattern of division has not been identified yet and what is valid for other species may result slightly or, on the contrary, considerably different for them. So, caution should be paid when discussing stem cells and, like in our case, the modality by which stem cells generate progenitor cells and thus the division mode. Other issues are about the provenience of stem cells and origin of the niche in Crustacea. Where do stem cells come from? How are they recruited? Were they already in place since embryogenesis or have they been displaced from one place to the niche? And what about the niche itself? Was it already in place since the neurogenesis or has it been built before the beginning of adult life? Is it a new structure? Where does it come from? Which are cells responsible for constructing the niche? All these questions still need explanations and are at the base of my project.

5.1.9. To sum up

From these studies, some general conclusions about the niche can be draw (Fig. 5.13): first of all, the structural asymmetry of the niche to guarantee that one of the two daughter cells stay in the niche and the other leave and differentiates. In fact, a unifying idea is that stem cells produce daughters with equivalent potential and their subsequent location relative to the niche determines their fate. Second, the tight connection between

the stem cells and a specialized cell group (cap cells, terminal filament cells) by cadherin ensures long-term self-renewal and third intrinsic factors and signaling cascades are mediate by the niche (Scadden, 2006).

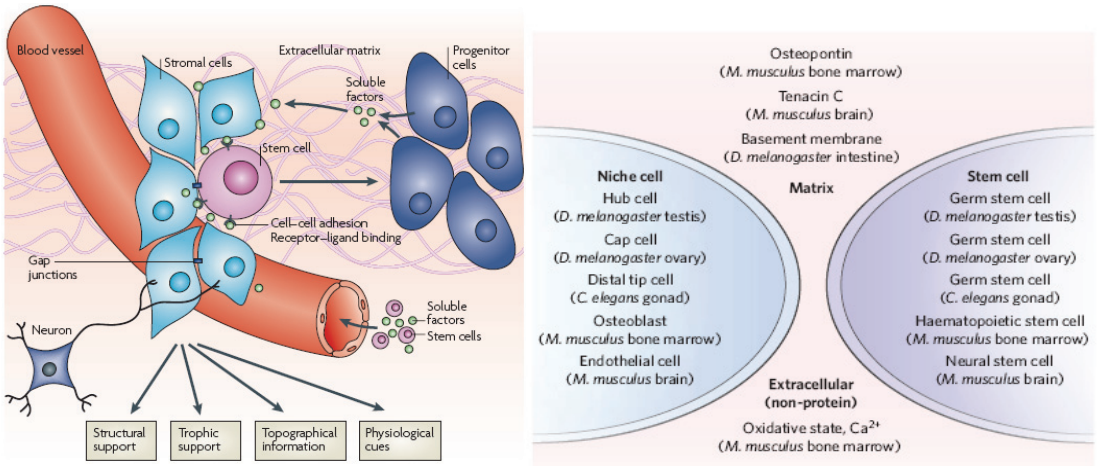


Fig. 5.13- On the left, a review scheme on componenets and function of the stem cell niche (from Jones and Wager, 2008). On the right, elements identified in stem cell niches from different organisms. (from Scadden, 2006).

So, it comes now crucial to fully understand the structure of the niche, the surrounding cells that form the main structure of the niche and its development. In my project on Marbled crayfish, by tracing the appearance of the niche back to embryogenesis, I aim to discover the specific time when the niche appears. The main purpose of the project is to follow the single steps which lead to the complete formation of the niche structure and to the time when the niche starts functioning, in order to value whether or not the embryonic-NB based mode of neurogenesis stops to function, if it stops at all.

5.2 RESULTS

5.2.1. Embryonic development of the brain

Embryos of different stages, from early to later development (according to the staging system described in Seitz et al. 2005), were incubated in BrdU for 4 hours. Detailed results of these early stages have been shown in Chapter 2. To summarize the general pattern of neurogenesis, the mitotic activity of neuronal stem cells was most intense between 38% and 42% of embryonic development (Pl. 5.1A, B, C). Some of these neuroblasts (NBs) can be identified individually based on their position within the developing brain and by their size and proximity to the progeny. In particular, on the ventral side of the brain the presence of a group of NBs (named "rosetta") is a feature of the deutocerebrum (Pl. 5.1). This type of peculiar arrangement ("rosetta") represents a "proliferative zone" made of neuroblasts and progeny that most likely will further develop into the **Deutocerebral Proliferative System** (DPS) in post-embryonic brains (Pl. 5.10B). At about E90-95%, mitotic cells are still present all over the brain, especially in the optic lobes (Pl. 5.2A, B). In the brain, EdU-positive cells (EdU+ cells) are arranged in scattered clusters, especially along the midline that separates the two halves of the brain (Pl. 5.2C, D, E). In fact, single NBs are still active and surrounded by their progeny. In the deutocerebrum, our region of interest, cells of different size classes are EdU+. They may represent both NBs and their progeny that, contrary to the progeny along the midline that remains tightly associated to their NB of origin, becomes dispersed and does not show any particular pattern (Pl. 5.2C, D, E). So, at the end of embryogenesis some NBs, along the midline, show a local proliferation because of a small progeny associated to them, some others are among a cluster of diverse size cells that form the primordial Deutocerebral Proliferative System (Pl. 5.2F; Pl. 5.3; Pl. 5.10B).

Just before and during hatching, the dispersed cluster of proliferative cells in the deutocerebrum starts to stretch towards the middle of the brain, to form a kind of band which characterizes the early post-embryonic phase of brain development (Pl. 5.4). The band appears contiguous proving the origin of the band from the primordial deutocerebral proliferative cluster (Pl. 5.4A, B, C, D). Immediately after hatching the shape of the proto-niche emerges among the mitotically active cells (Pl. 5.4E-F-G) and is located adjacent to the giant neuron. It is possible to notice the reduction in number of NBs,

dispersed in the brain, from end of embryogenesis (Pl. 5.4A) to POI stage (Pl. 5.4D). The development of the niche will be shown in the following images.

5.2.2. Between end of embryogenesis and hatching stages: the appearance of the niche

At the end of embryogenesis, immediately before hatching, some NBs are still present in the brain. On the ventral surface of the brain, labeled by tyrosinated tubulin, a bunch of filaments appears together with fibers that wrap single cells of a still not defined group of cells (Pl. 5.5B-C-D). The initial bunch of filaments emerges close to the giant neuron (GN) that from now on will be considered the landmark for identifying the position of the niche (Pl. 5.5A). On the ventral side of the brain, in the deutocerebrum, some clustered cells, close to the giant neuron, appeared EdU+ which means that they are either ready to divide or they represent progeny of cells (Pl. 5.5A). Some NBs are still present and groups of cells (most likely single NB associated with progeny) are more or less patchily dispersed in the brain (Pl. 5.5A). The initial group of cells associated with the forming filaments is labeled by the nuclear staining YOYO (Pl. 5.5B, C, D). Cells appear not homogenous in terms of sizes and shapes and are not distinct from other cells at the ventral surface of the brain (Pl. 5.5B, C, D).

Development of the filament core continues, filaments appear to converge to a point or central pore which is made visible by the intense tyrosinate tubulin signal of the single filaments (Pl. 5.6). The area around the aggregating filaments is a continuous with the rest of the brain surface, without any boundaries that define a specific structure (YOYO, nuclear staining. Pl. 5.6C, D). However, a band of EdU+ cells in the region close to the filamentous structure represents the first signs of the proto-niche from the deutocerebrum (Pl. 5.6A, B).

Often, when analyzing embryos, it is possible to observe a difference time schedule of the appearance of the bilaterally paired niches that will show up in each halves of the brain (Pl. 5.7A). One niche may develop earlier than its counterpart and be already discernible as archetype of the niche (**proto-niche**) (Pl. 5.7A left side of the brain) while the other one in still in its infancy (Pl. 5.7A, right side of the brain; Pl. 5.7B).

Now, the embryonic phase can be considered completed, the embryo is now about to

hatch and the proto-niche is more definite. Double labeling with the nuclear staining YOYO and tyrosinated tubulin highlighted the whole structure of the protoniche (Pl. 5.8) which can be already subdivided into two main regions, each of them characterized by two different types of cells. One region surrounds the filamentous part of the proto-niche and is made of smaller and spindle-shaped cells (Pl. 5.8B, C). A second region encircles the previous one and is made of spherical cells (Pl. 5.8C). A closer look at the forming fibrous core is shown in Fig 9A and B. Filaments converge parallel to one end, forming a kind of channel-structure (the **pore**). Surrounding EdU+ cells suggest an intense cell cycle activity during the first post-embryonic stage of the animal (POI). The POI phase may last up to three days (Seitz et al., 2005) and during this stage the proto-niche reaches a more compact and defined organization that, together with the surrounding structures to which it is always connected (stream, LPZ, cluster 10), forms the Deutocerebral Proliferative System (Pl. 5.10B). The proto-niche can now be clearly subdivided into two main parts: a hub of cells (the "**niche-core**") wrapped by filaments that form the pore, and a multi-layer of proliferative cells (the "**cap**") on top of the core (Pl. 5.10B). Once again, two types of cells can be distinguished: spindle shaped cells forming the niche-core, and dividing rounded cell forming the cap (Pl. 5.10A). Some neuroblasts (identifiable since embryogenesis) are still present in the brain (Pl. 5.10A). In the cap, one large Edu+ cell is undoubtedly associated to smaller cells, two of which appear to have just divided symmetrically (Pl. 5.10A).

Later in the POI stage, the cap and the niche-core have acquired a definite spatial structure (Pl. 5.11). The joining band between the proto-niche and cluster 10 is thinner and cells are lined up like pearls on a string (Pl. 5.11). Contrary to the niche-core which is labeled by only tyrosinated tubulin, cells of the cap are Edu+ and three types of size classes can be arbitrarily distinguished. Here we name them **Type1**, spherical mother cells; **Type2**, medium daughter cells; **Type3**, small progeny. Relationship of these cells ("mother", "daughter", "progeny") is purely based on a size criterion. As said above, the cap contains cells of three size classes: Type1 and Type2 are on the ventral side of the cap (Pl. 5.11A), Type3 are located more dorsally (Pl. 5.11B). A count and area-measurement of EdU+ cells of the cap of proto-niches at the same developmental stage (POI) showed a distribution of the three classes that is not uniform (Pl. 5.12). Cell areas

may range from 40 to 300μm^2, some proto-niche shows a certain uniformity of distribution of cells (Pl. 5.12A, B, E), some others include only cells of a definite area size, with no intermediate cells (Pl. 5.12C, D).

During the POI stage, few neuroblasts are still present in the brain (Pl. 5.13). In each of the processed samples, cap cells incorporate label after incubation of 4 hours in EdU. By contrast, in only few cases did the niche-core contain some EdU+ cells (Pl. 5.13). To understand whether these EdU+ cells derive from a local proliferation of the niche-core or they derive from the cap, I performed a pulse chase experiment by using two different mitotic markers (BrdU **first pulse**, Edu **second pulse**) applied in successive pulses separated by a 48 hours interval (Pl. 5.14. See Pl. 5.6, Material and Methods, Chapter 6). This type of experiment suggested that some of the progeny (BrdU+ cells) born 48 hours earlier by the cap cells (EdU+ cells) moved into the niche-core, after their birth (migration of cells is based on the hypothetical mechanism of the niche proposed by Sullivan et al., 2007). Some other BrdU+ cells remained in the cap, close to Type1 cells (Pl. 5.14B).

5.2.3. The function of the niche: the pulse-chase experiment

A series of pulse-chase experiments (Pl. 5.6, Material and Method, Chapter 6) was performed to understand when the niche starts working and which cells are involved.

The first pulse-chase experiment was executed immediately before hatching, this means that animals expected to hatch the following day were incubated in a first pulse of BrdU for 4 hours. During the 24 hours interval between the two incubations, animals hatched to POI stage. Thus, the second pulse in EdU was applied to these POI stages. By doing so, it was possible to catch the very first signs of activity of the niche which is still forming and showing only the first outline of the fibrous core (Pl. 5.15A, D). The proto-niche is not visible (as it was in previous images of the same stage), but the bunch of filaments as detectable by the tyrosinated tubulin staining emerges close to one single mitotically active cell highlighted by the two markers: BrdU (Pl. 5.15C, D) and EdU (Pl. 5.15B, D) and also by the tubulin staining. From now on this cell will be named **Neuroblast-X (NB-X)**, the only identified cell that has been found so far associated to a chain of newborn cells. In no other cases, in the current project, single EdU/BrdU+ cells have

been found associated to a chain of numerous newborn cells. The identity of NB-X, NBs and in general S-phase cells will be treated in the Discussion. The overview of the proto-niche, filaments and NB-X is shown in Pl. 5.15D. At this stage (end of embryogenesis-POI), new born cells from NB-X either remain in place (Pl. 5.15A) or are sent off along the "migratory stream" (according to the model proposed by Sullivan et al., 2007a) towards the forming cluster 9 (Pl. 5.15C, D). In later POI, while the niche develops, the migratory stream between the niche and LPZ becomes thinner, cap cells proliferate, Neuroblast-X is adjacent to the proto-niche and, together with some cap cells (most likely Type 1 and 2), contributes to the production of progeny towards cluster 9 (Pl. 5.16). This mitotic activity was demonstrated by the pulse-chase experiment. BrdU+ cells (first pulse) from the cap and NB-X ended up in cluster 9, the cluster hosting the somata of local olfactory interneurons. In cluster 9 there are exclusively BrdU+ cells, not EdU+ cells (second pulse) (Pl. 5.18A, B, D). An overview of the deutocerebral proliferative system together with NB-X is provided in Pl. 5.17A. Higher magnifications of NB-Xs are also shown (Pl. 5.17B, C, D). The niche is not visible because it is located more dorsally. The pulse-chase experiment was repeated several times on several animals with the same result: while the niche takes shape and the cap maintains its continuous dividing activity, newborn cells (Pl. 5.18A, B, D) are added to cluster 9 by migration (according to the model proposed by Sullivan et al., 2007a) along a stream which connects the niche to cluster 9. The NB-X is well visible in all preps at the POI stage (Pl. 5.18A, B, C).

The same pulse chase experiment showed that the mitotic activity in the LPZ was also significant (Pl. 5.19, 20). From the current data, three zones could be distinguished in this area of the Deutocerebral Proliferative System (DPS), based on the presence on BrdU+/EdU+ cells: LPZ (both BrdU+ and EdU+ cells), cluster 10 (no BrdU+ or EdU+ cells), and a third zone in between (the **Transit Zone**, **TZ**) with only BrdU+ cells. Not in all preps this scenario was reproducible, the classification in three zones is merely a question of simplicity in suggesting a putative mechanism of translocation of newborn cells from LPZ to the most peripheral zone (cluster 10). In detail: the most peripheral zones of DPS (cluster 10), no cells in S-phase is present (Pl. 5.20A). The transit zone contains some dispersed BrdU+ cells (Pl. 5.19B). The LPZ showed a mixture of EdU+ and BrdU+ cells (Pl. 5.19A, B, C).

Higher magnifications of the LPZ are shown in Pl. 5.20. The LPZ is made of two or three bigger cells which show mitotic activity during the post-embryonic and adult life of crayfish. These proliferating cells are located at the proximal part of the stream, towards cluster 10, which looks enlarged (Pl. 5.19B, C and Pl. 5.20). The pulse chase experiment gave evidence of the relationship between these different size classes of cells. Type1 cells are labeled by EdU while the progeny (Type2 and 3) were positive to BrdU (Pl. 5.20G). Two symmetrical cells (Type2) tightly associated to the mother bigger cells represent the progeny. Same scenario is visible in Pl. 5.20H where Type1 cells are associated to Type3 cells close to the Type2.

5.2.4. POII stage: the detachment of the cap

While the animals undergo a second molt to the POII stage, the niche takes a further step in its progress as well. The cap is clearly distinct from the niche-core (Pl. 5.21A, B) and starts to detach from it (Pl. 5.21C, D). The stream towards the LPZ is now completely separated from the cap. It is attached to the niche-core itself and enlarges towards the LPZ. Some NBs are still dispersed in the brain, the NB-X is not anymore visible. No data are available about its fate. During the POII stage, the cap gets displaced from the niche-core towards cluster 9 (Pl. 5.22A, B). Now the niche is not anymore close to the giant neuron, but placed in the middle between the cap and LPZ. EdU+ cells appear within the niche, at its lateral endings, almost symmetrically (Pl. 5.22C, D). By pulse-chase experiment, it was possible to show the movement of newborn cells and the local mitotic activity of the resident stem cells (see Discussion for the term “stem” cells) (Pl. 5.23). In the POII stage, the cap has moved away from the niche which now looks isolated and between the cap and LPZ (Pl. 5.23). The cap still contains EdU+ cells, and appears like a continuous line towards cluster 9 (Pl. 5.23).

5.2.5. Juvenile (14mm body length)

In juveniles, the structure of the niche was labeled with gluthamine synthetase, a glia cell marker, and cells in S-phase were visualized by BrdU.

In some specimens, the cap appears just detached from the niche (Pl. 5.24A), whereas in others the cap is already displaced from the niche to reach cluster 9. The cap becomes

what, in adults, is called MPZ (medial proliferative zone) (Pl. 5.24B, Pl. 5.25A, B). A couple of dividing cells are visible in the niche-core (Pl. 5.24A) and many BrdU+ cells are packed and wrapped by filaments within the cap/MPZ (Pl. 5.25A, B). Migratory streams which link the niche-core to both LPZ and MPZ are thin and no BrdU+ cells are labeled within these streams. However, within the niche-core, one or two BrdU+ cells are present in almost all preps (Pl. 5.26A-C). It is interesting to notice the presence of a pool of gluthamine synthetase+ cells located in proximity of the niche (Pl. 5.26A and 27). These cells have two lateral cytoplasmatic extensions (Pl. 5.27A, B). Another interesting observation is that the migratory stream has shrunken in size during the development of the deutocerebral proliferative system (Pl. 5.28A, B). Towards the end of embryogenesis and in the early POI stage, the stream was more like a band fully packed with cells, while towards end of maturation of the niche (juvenile stage) the migratory stream looks more like a thin string connecting the niche-core to the two proliferative zones (Pl. 5.28A, B).

5.2.6. The glia hypothesis (Sullivan et al., 2007): labeling by gluthamine synthetase

In order to verify whether the niche cells have glial characteristic, the development of the niche from embryonic stages to juveniles was monitored by using gluthamine synthetase (a glia cell marker), instead of tyrosinated tubulin. To visualize the development of the olfactory and accessory lobes, a probe for actin was used. In crayfish embryos, at around E80%, olfactory and accessory lobes start to emerge in the deutocerebrum (Pl. 5.29A, B). As development continues (E85-E90%), the lobes become bigger in size (Pl. 5.30A,C) while, on the ventral surface of the brain, cells stained by gluthamine synthetase aggregate to form a more compact group of cells (Pl. 5.30B, D) that changes in shape through the final stages of embryogenesis (Pl. 5.31A-C). During POI, the pore becomes visible also with gluthamine synthetase (Pl. 5.31D) which stains most of the cells in the brain (Pl. 5.31). In POII (Pl. 5.31E), it is noticeable that the cap is detaching from the niche-core and in juvenile (Pl. 5.31F) the niche appears in its final structure.

5.2.7. Development of the niche in lobster

A comparative study with the American lobster (*Homarus americanus*) was done in order to understand whether a deutocerebral proliferative system (DPS) was present as well.

The female lobster can release up to 10,000 eggs but only 10% survive (Pl. 5.32 A, B). After hatching, lobsters go through four larval stages separated by successive molts (Pl. 5.32 C, D). Larvae IV are the ones that resemble the adult shape. By this time the animal is about one month old and this stage may last several weeks (Pl. 5.32 D).

Most of the results presented here come from second and fourth stage larvae. Animals were processed similarly to Marbled crayfish but some of the antibodies successfully used in crayfish did not work in lobster. For these reasons, images from gluthamine synthetase and tubulin are not available, as well as images of the niche itself. Nevertheless, a proliferative system was found. Streams of newborn cells from a group of dividing cells, placed between the proliferative zones (LPZ and MPZ), on the ventral surface of the brain are present. In the deutocerebrum, after incubation of larvae in BrdU, the two lateral proliferative zones (LPZ and MPZ) are clearly discernible, together with two streams of cells which appear to converge to one point in between (Pl. 5.33 A-C, white circle). The situation is not as clear as in the crayfish in which the structure of the niche undoubtedly appeared as part of the system. In lobster, gluthamine synthetase (Pl. 5.33) did not work, neither tubulin. A closer look at the associated cells placed in between the two proliferative zones is provided (Pl. 5.34). In II stage larvae, three bigger cells stand out among others which look smaller and associated to them (Pl. 5.34A, B). Images from different animals of the same stage showed a similar scenario in which two or three bigger cells are associated as a group placed along the convergence of the two streams and smaller cells are seen to derive from them (Pl. 5.35A). In some other cases, the bigger cells appear as a group with smaller associated cells that, in turn, form a contiguous line with the departing streams. The whole system may resemble the one seen in crayfish (niche) but more experiments with more specific antibodies are needed.

5.2.8. Plates

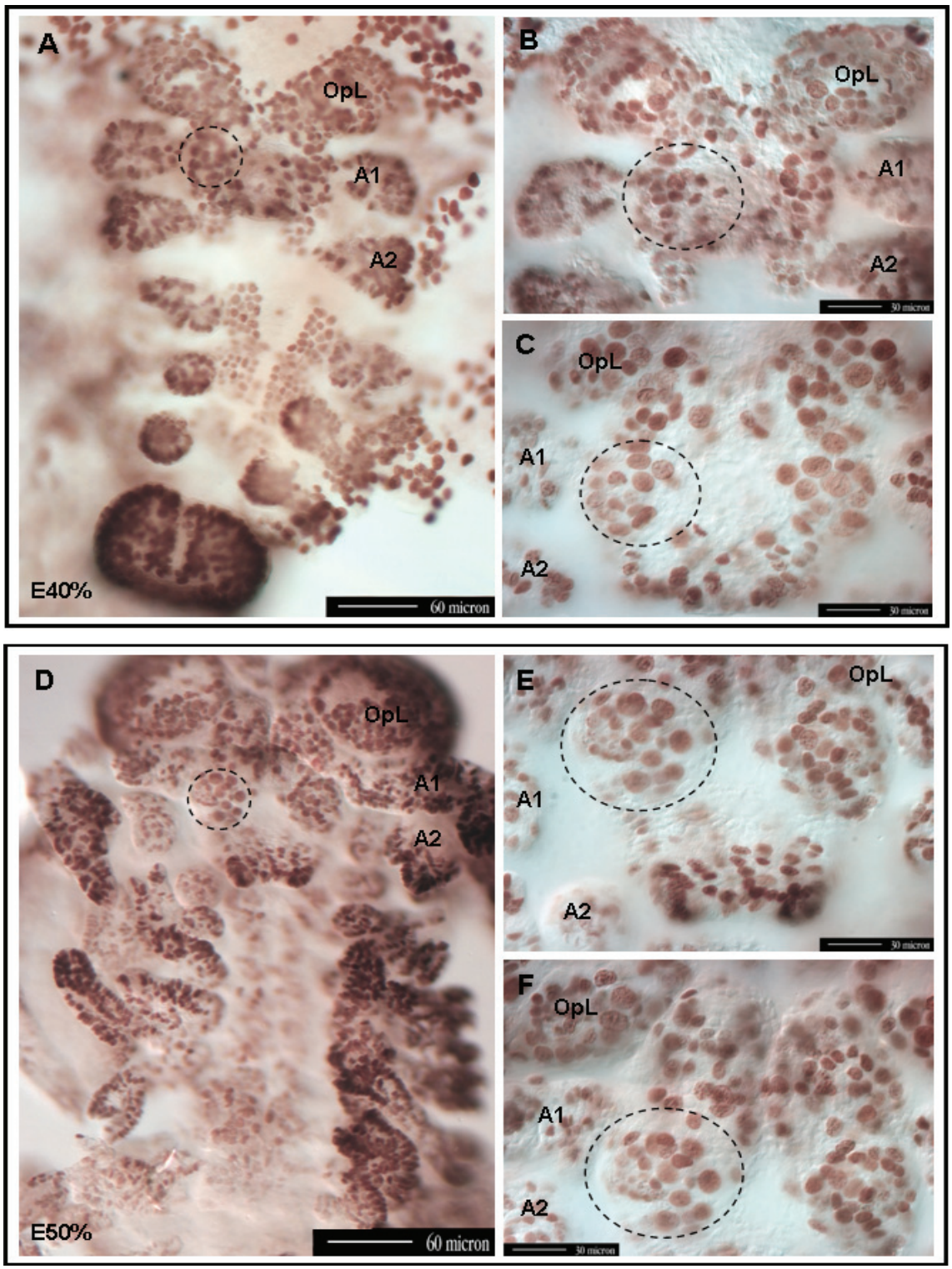


Plate 5.1- BrdU staining in embryos of the Marbled crayfish at 42% (A-C) and 60% (D-F) of embryonic development (E42% and E60%). A, D: whole embryo; B, C, E, F: brains. Abbr: OpL, optic lobes; A1, antenna I; A2, antenna II. "Rosetta" (dotted circle).

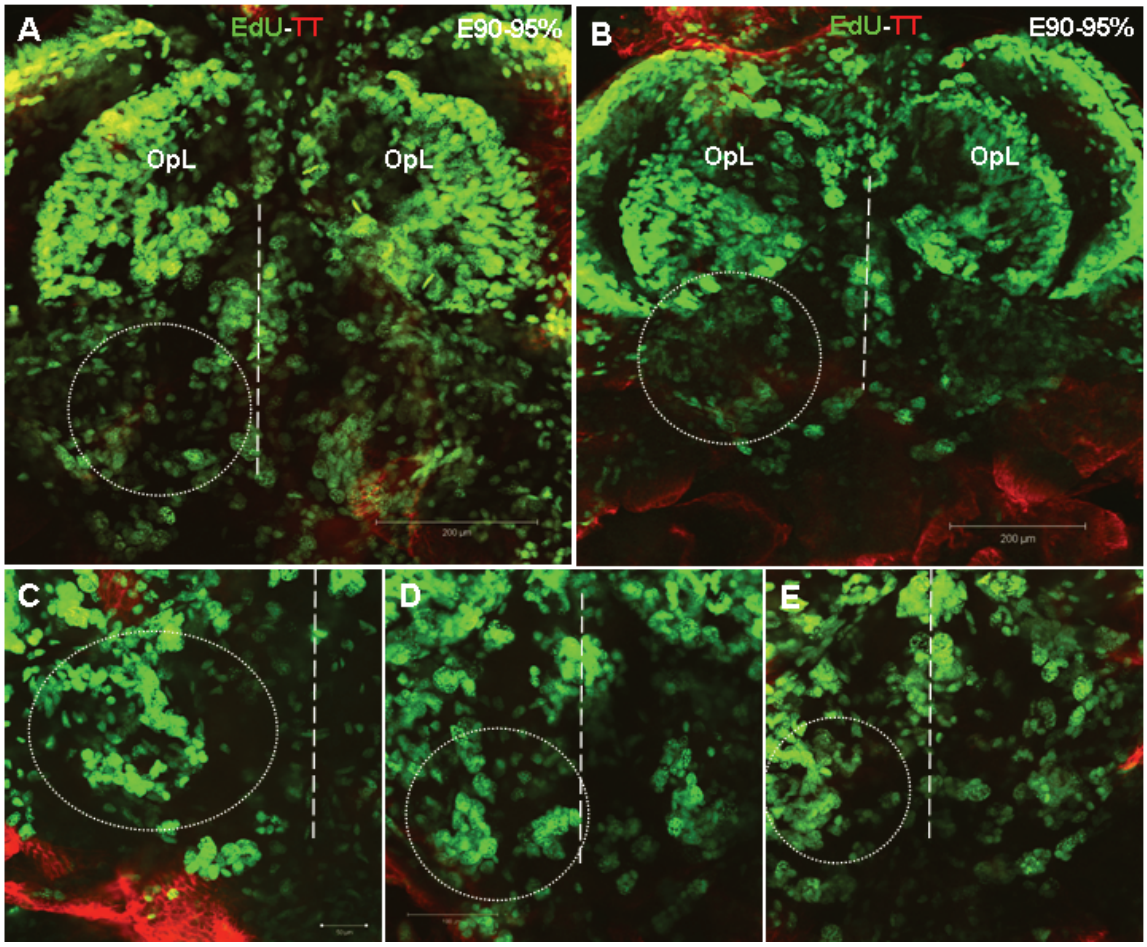


Plate 5.2- Mitotic activity in the brain of the Marbled crayfish at about E90-95%. A, B: brains at about E90-95%, double staining with EdU and Tyrosinated Tubulin (TT). C-E: higher magnification of the brain: deutocerebrum (dotted circle); midline (dotted line). Abbr: OpL, optic lobe.

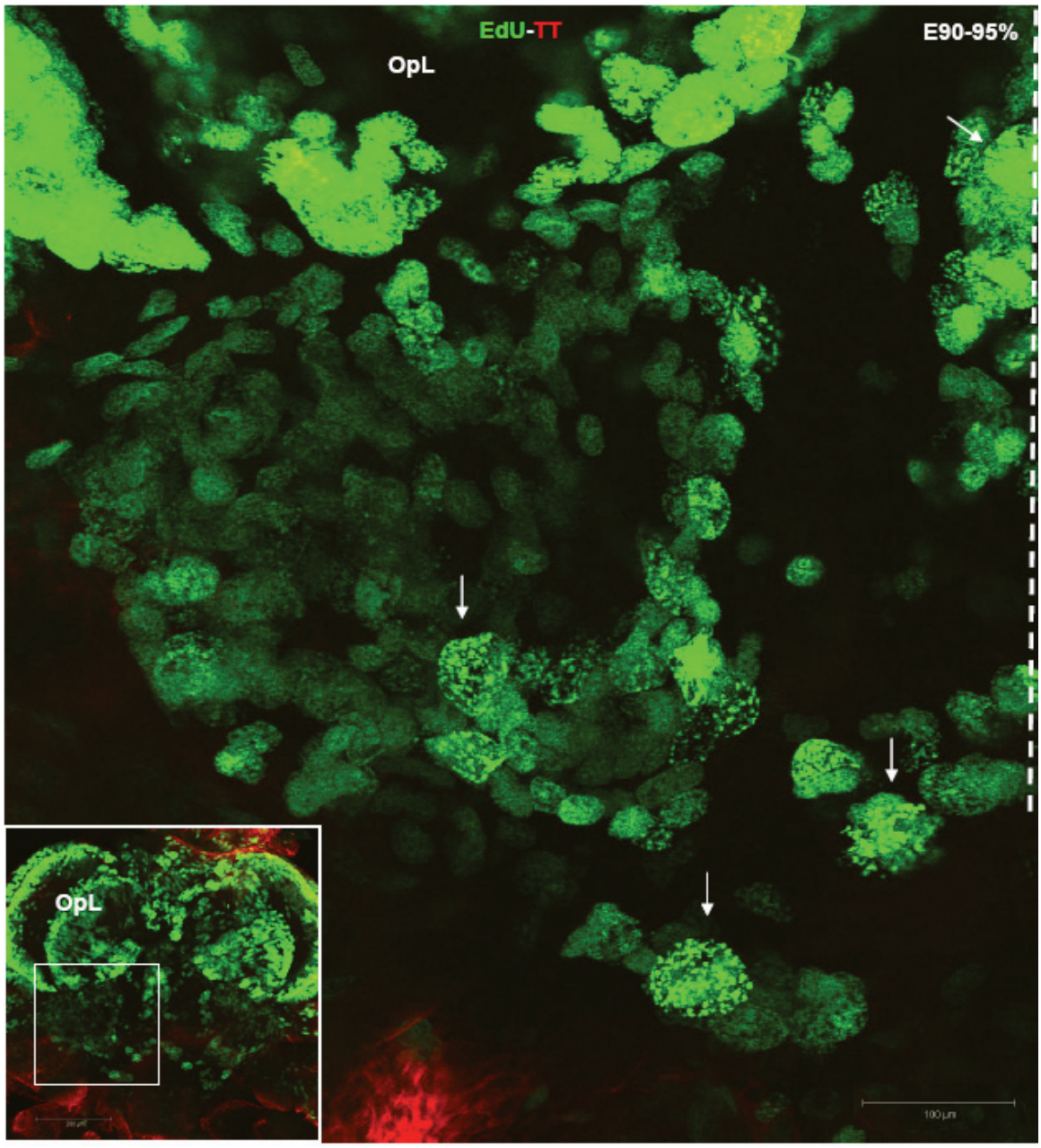


Plate 5.3- Mitotic activity in the deutocerebrum of the Marbled crayfish at about E90-95%. Double staining with EdU and Tyrosinated Tubulin (TT). EdU+ cells of different sizes are present, mostly NBs (neuroblasts, white arrow) and their progeny. Midline: dotted line. Inset: overview of the brain at E90-95%. The deutocerebrum is highlighted by the square. Abbr: OpL, optic lobe.

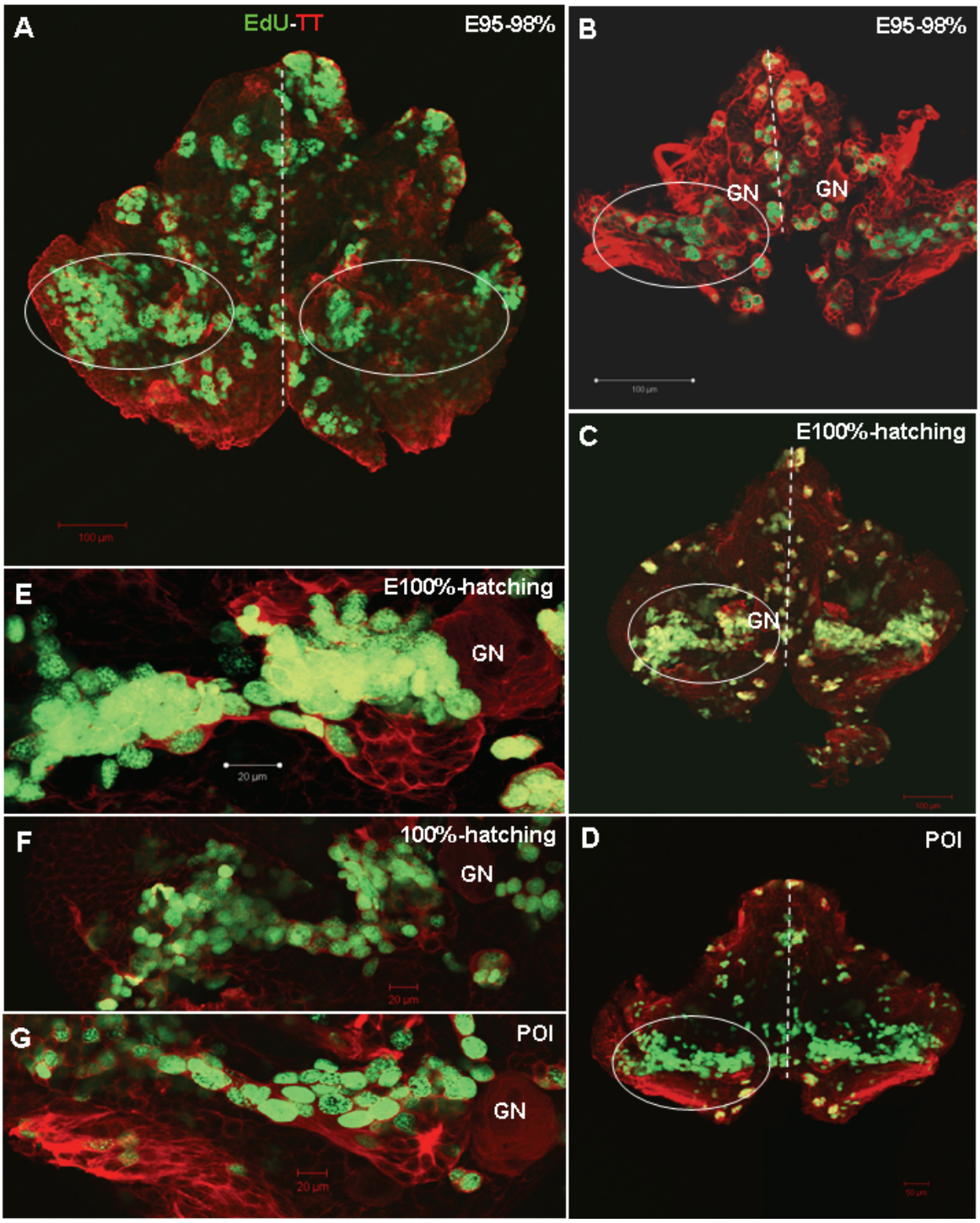


Plate 5.4- Mitotic activity in the brain and deutocerebrum of the Marbled crayfish, double staining with EdU and Tyrosinated Tubulin (TT). A-D: development of the brain from E95-98% (A, B), through hatching (C) to POI (D): a cluster of EdU+ cells (circle) in the deutocerebrum stretches towards the middle of the brain (dotted line). Note the reduction in number of NBs, dispersed in the brain, from end of embryogenesis (A) to POI stage (D).E-G: overview of the EdU+ cells band that appears as a continuous from the cluster 10. Cluster 10, the band of cells and the forming proto-niche have been named: the Deutocerebral Proliferative System (see text for explanations). Abbr: GN: giant neuron.

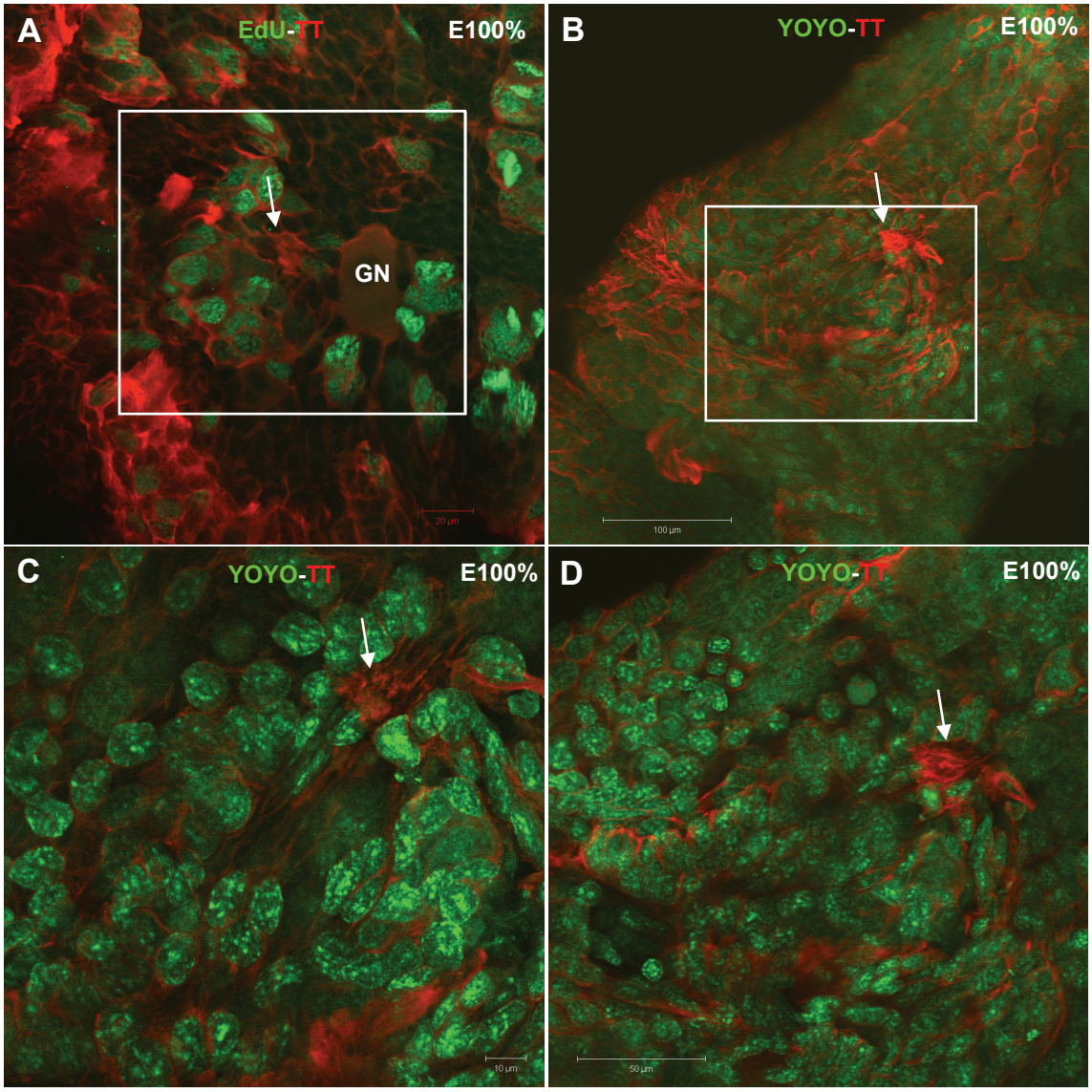


Plate 5.5- Overview of the deutocerebrum of the Marbled crayfish at the end of embryogenesis (E100%). A: double staining with EdU and Tyrosinated Tubulin (TT). B-D: double labeling with the nuclear staining YOYO and Tyrosinated Tubulin (TT). C, D: different optical sections that magnify the deutocerebral zone in B (square). On the ventral surface of the brain, a bunch of filaments (white arrow) shows up among cells of unidentified origin and identity and close to the giant neuron (GN).

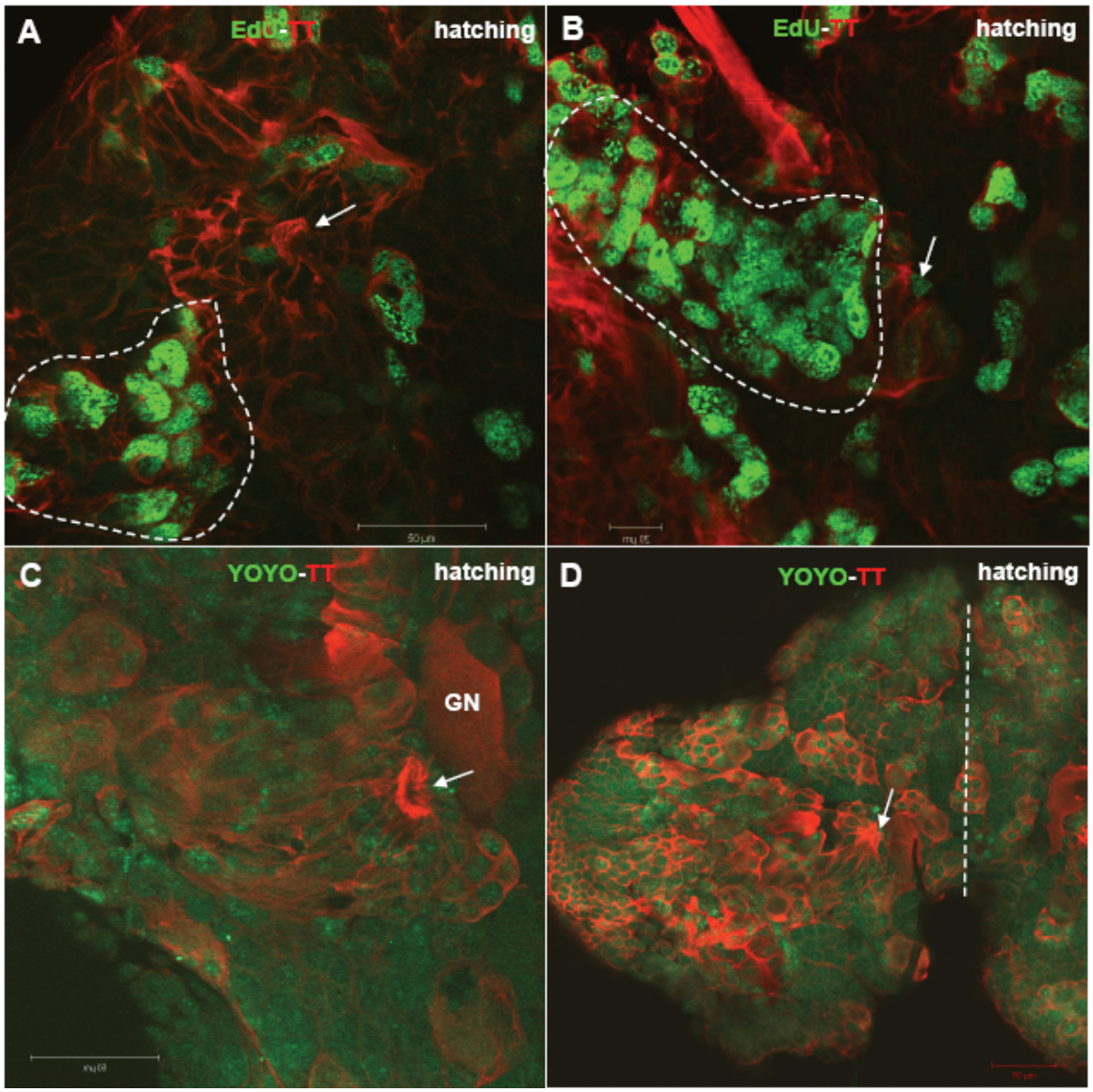


Plate 5.6- Overview of the deutocerebrum of the Marbled crayfish at the end of embryogenesis/hatching. A, B: double staining with EdU and Tyrosinated Tubulin (TT). C, D: double labeling with the nuclear staining YOYO and Tyrosinated Tubulin (TT). The filament core that characterizes the central part of the niche structure starts to develop (white arrow). Filaments converge and form a tip strongly labeled by tyrosinate tubulin. EdU-positive cells (A, B) form a band which evokes the origin of the forming proto-niche from the deutocerebrum.

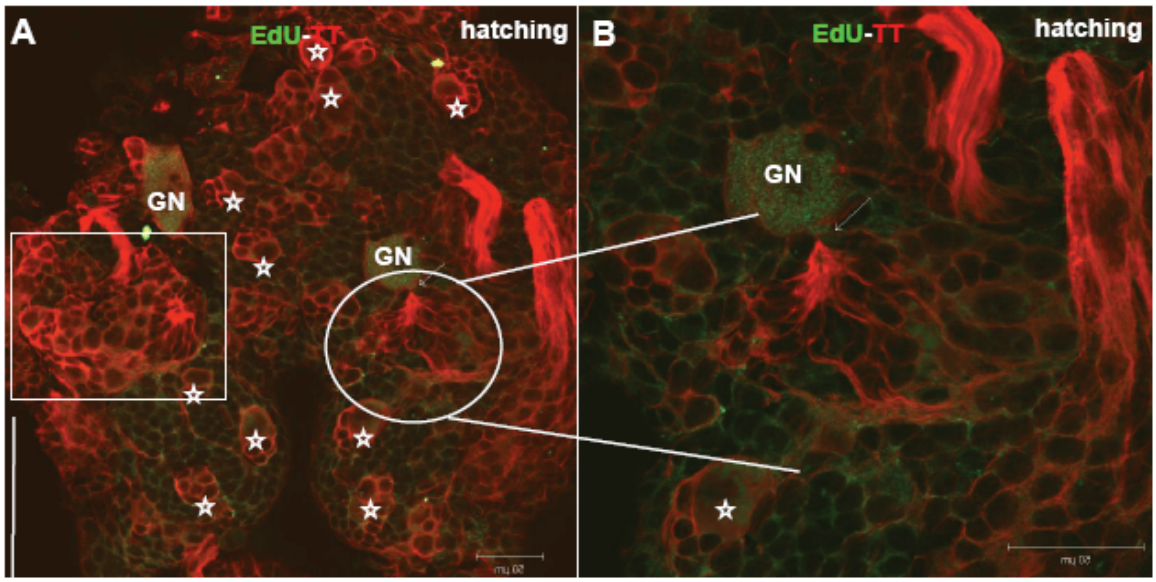


Plate 5.7- Overview of the deutocerebrum of the Marbled crayfish during hatching. A, B: double staining with EdU and Tyrosinated Tubulin (TT). A: two forming proto-niches are symmetrically located on the ventral surface of the deutocerebrum (square on the left, circle on the right). Note the different stage of maturation. In the brain, NBs are still patchily dispersed (white stars). B: higher magnification of a forming proto-niche, characterized a the tip made of filaments (arrow). Abbr: GN, giant neuron.

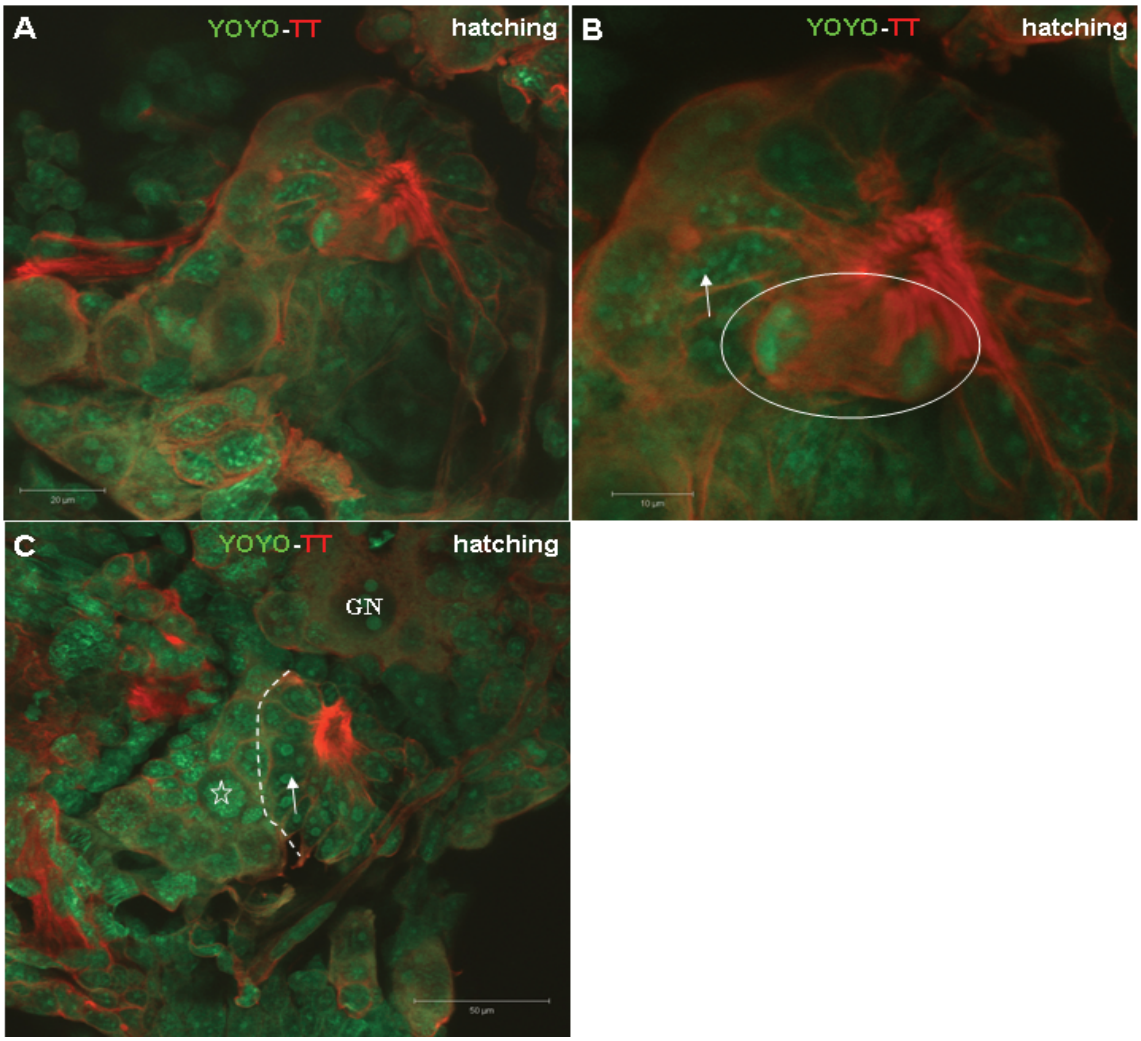


Plate 5.8- View of the forming proto-niche during hatching. A-C: double labeling with the nuclear staining YOYO and Tyrosinated Tubulin (TT). B: dividing cells appear in the zone surrounding the fibrous pore (white circle). This zone is made of spindle-like cells (arrow). C: two different types of cells can be distinguished within the forming proto-niche that later on will be formed by the cap and the niche-core: spindle-shaped cells around the forming fibrous pore (white arrow) and spherical-shaped cells (white star). The dotted line separates the cap from the niche-core. Abbr: GN, giant neuron.

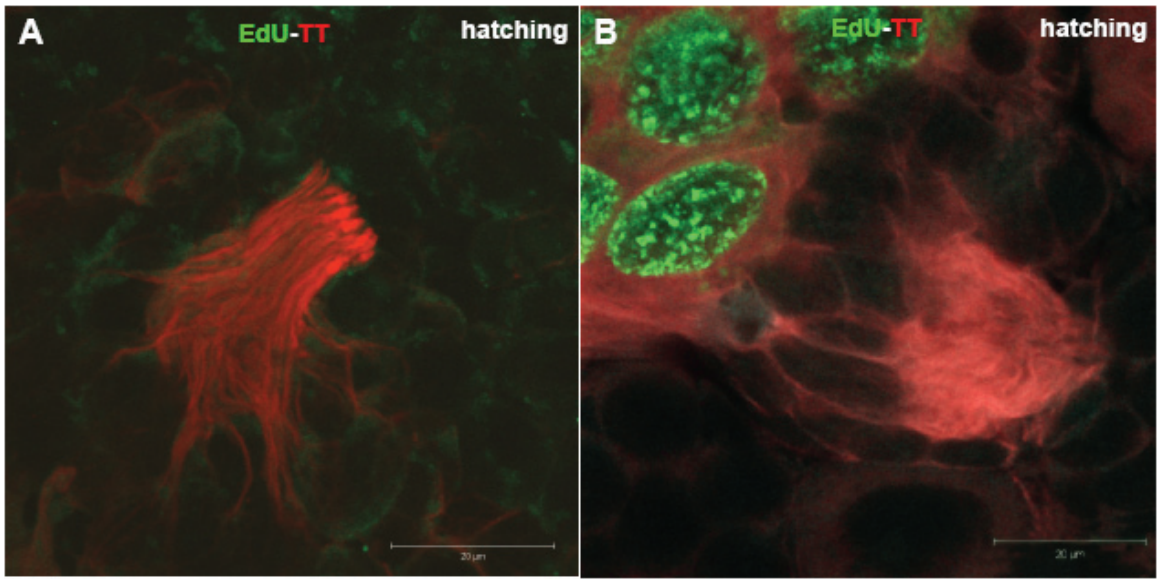
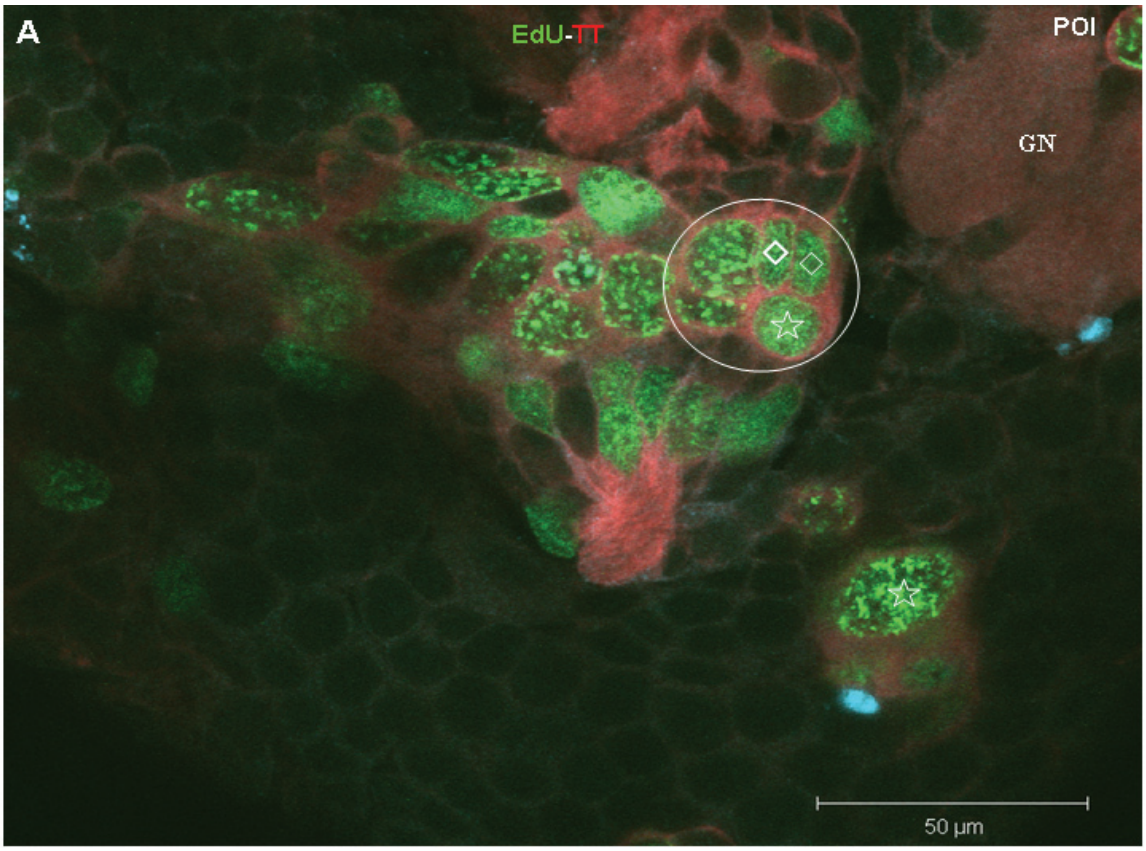


Plate 5.9- The fibrous pore surrounded by converging filaments. A, B: double staining with EdU and Tyrosinated Tubulin (TT). EdU+ cells form the cap pf the proto-niche.



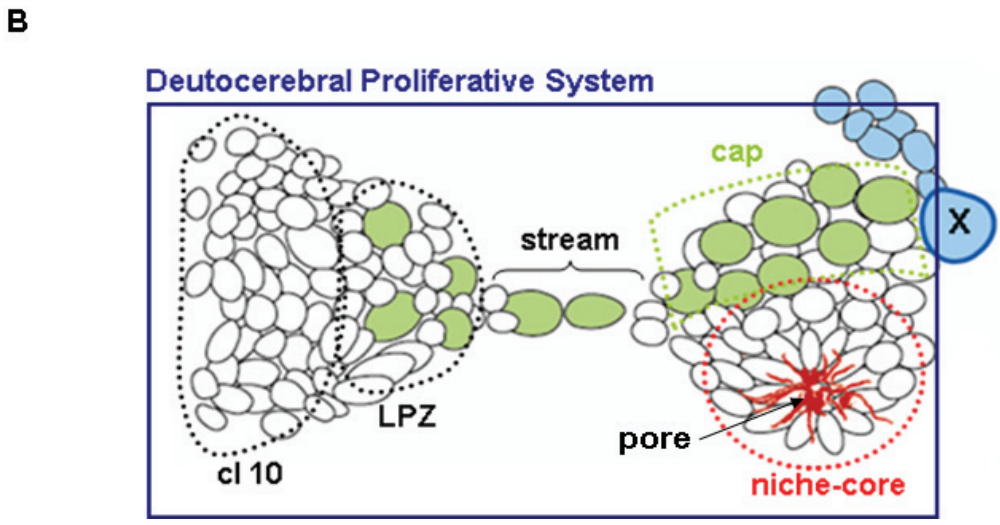


Plate 5.10- A: View of the proto-niche during POI stage. Double staining with EdU and Tyrosinated Tubulin (TT). The proto-niche is divided into two parts: the cap and the niche-core. Within the cap, round bigger cells (white star) are surrounded by smaller cells (white circle). Two of them are symmetrical, most likely after a mitotic division (white diamond). In the brain, some NBs are still present (white star). B: scheme of the Deutocerebral Proliferative System, made of the proto-niche (divided into the cap and the niche-core that surrounds the pore), the stream, LPZ and cluster 10. Abbr: GN, giant neuron, X, Neuroblast X, LPZ, lateral proliferative zone (see text for explanations).

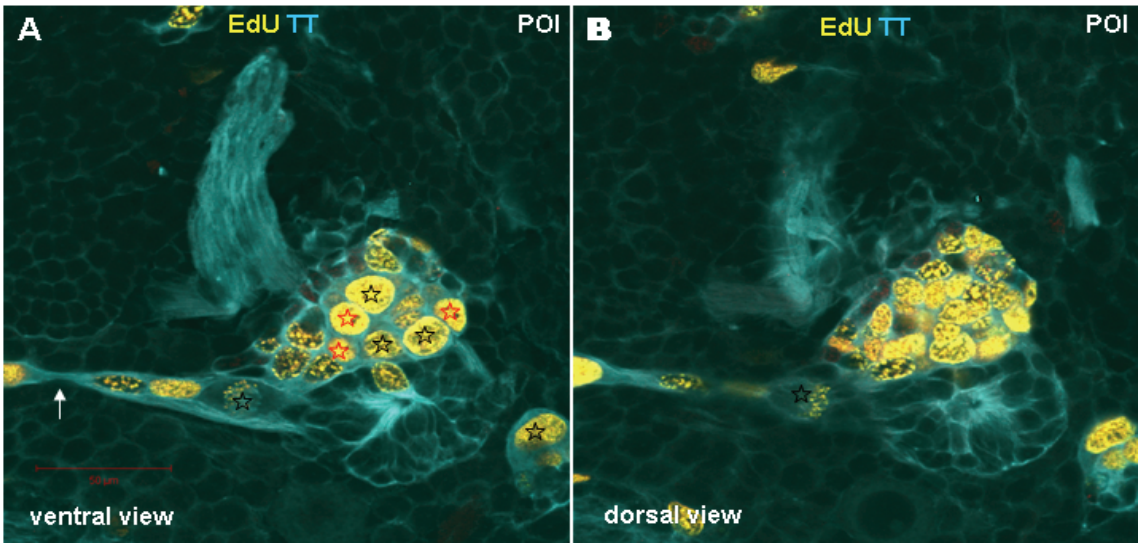


Plate 5.11- View of the proto-niche during POI stage. Double staining with EdU and Tyrosinated Tubulin (TT). A, B: single optical sections at different levels. The proto-niche is divided into two parts: the cap and the niche-core. A: within the cap, on the ventral side, round mother cells (black star) are discernible from medium size daughter cells (red star). B: the smaller progeny is dispersedly located more dorsally.

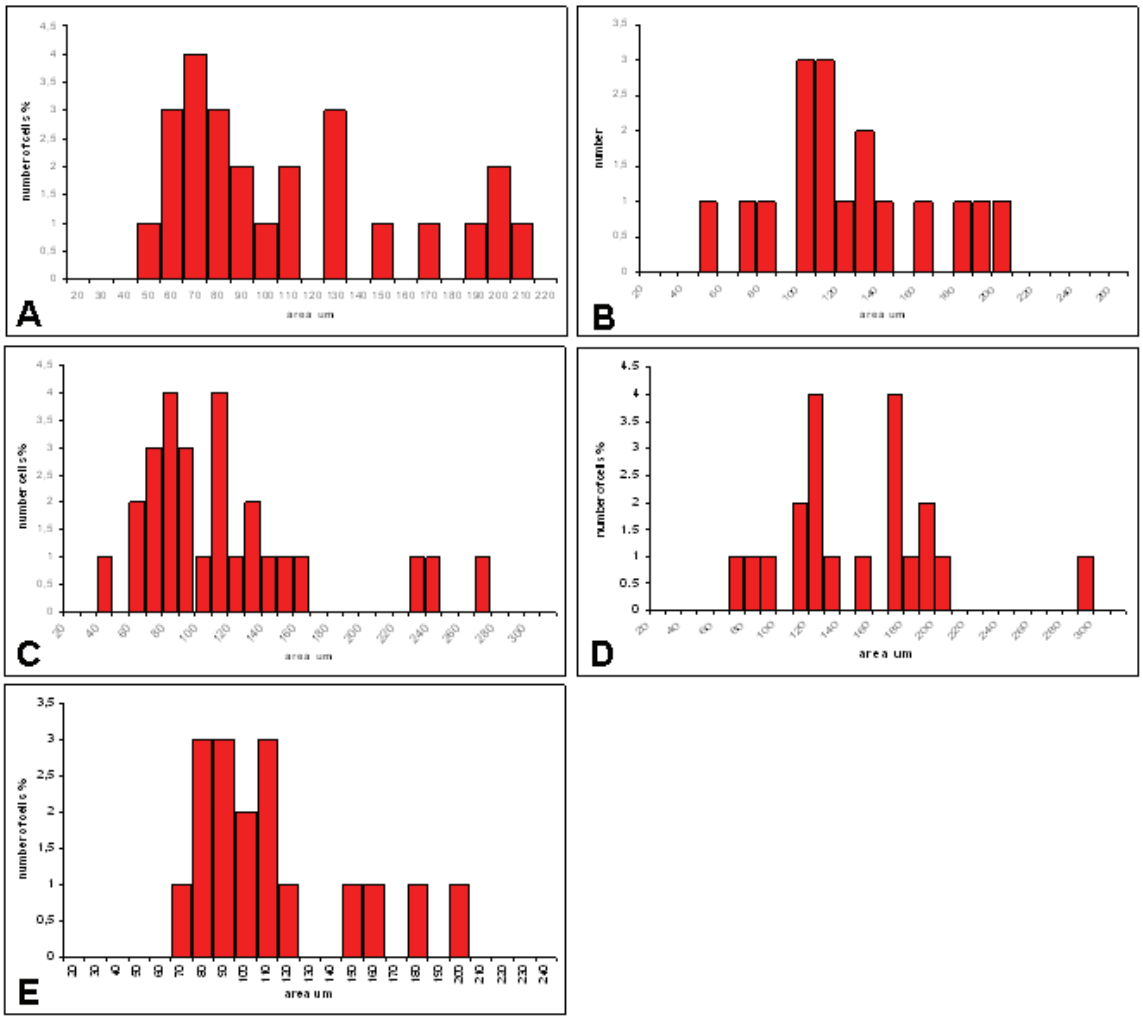


Plate 5.12- Histograms of size-classes of EdU positive cells, within the cap of the proto-niche, during POI stage.

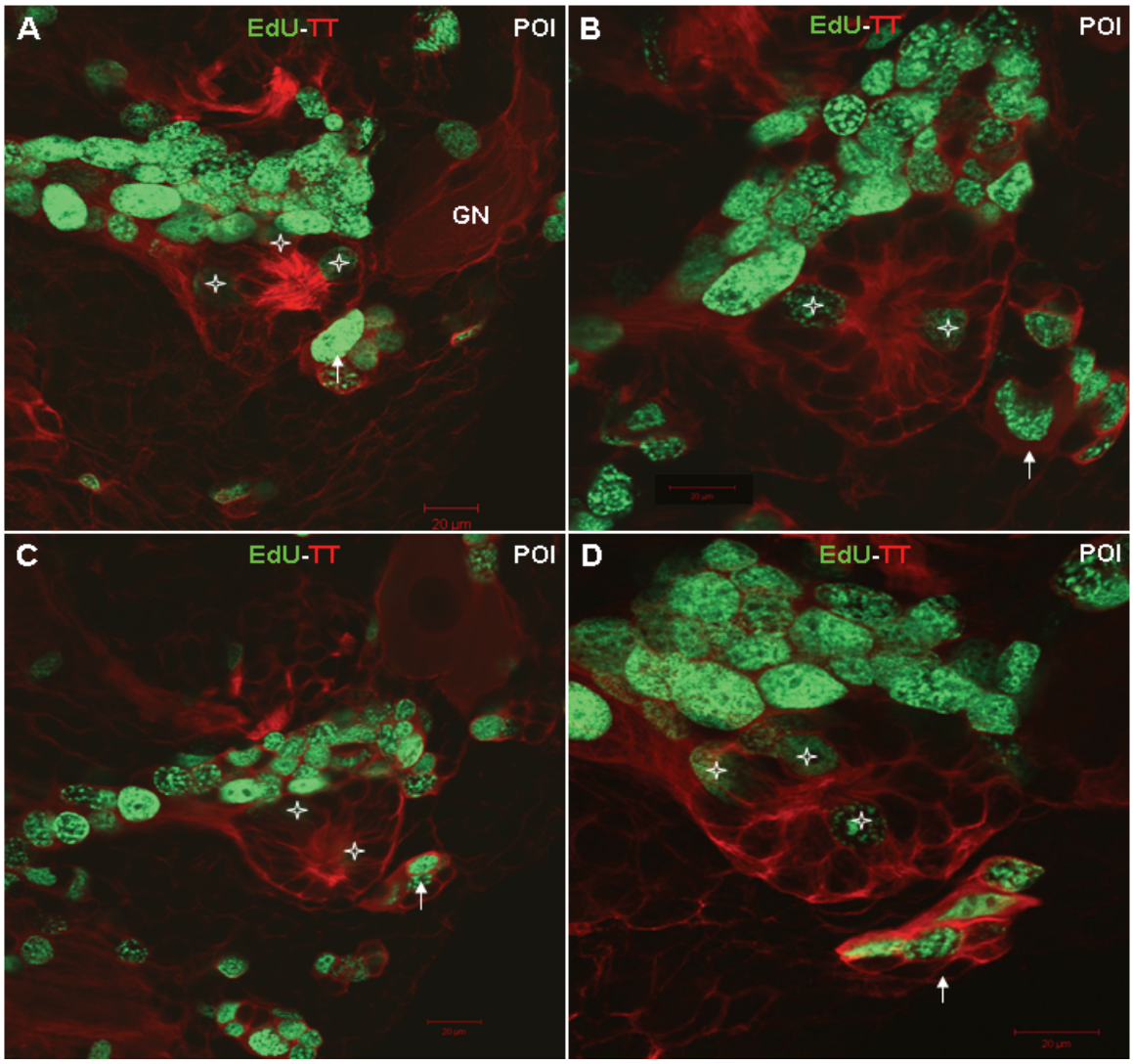


Plate 5.13- View of the proto-niche during POI stage. Double staining with EdU and Tyrosinated Tubulin (TT). In the niche-core, some cells are EdU+ (white asterisks), as well as the few left neuroblasts still present in the brain during POI stage (white arrow). Abbr: GN, giant neuron.

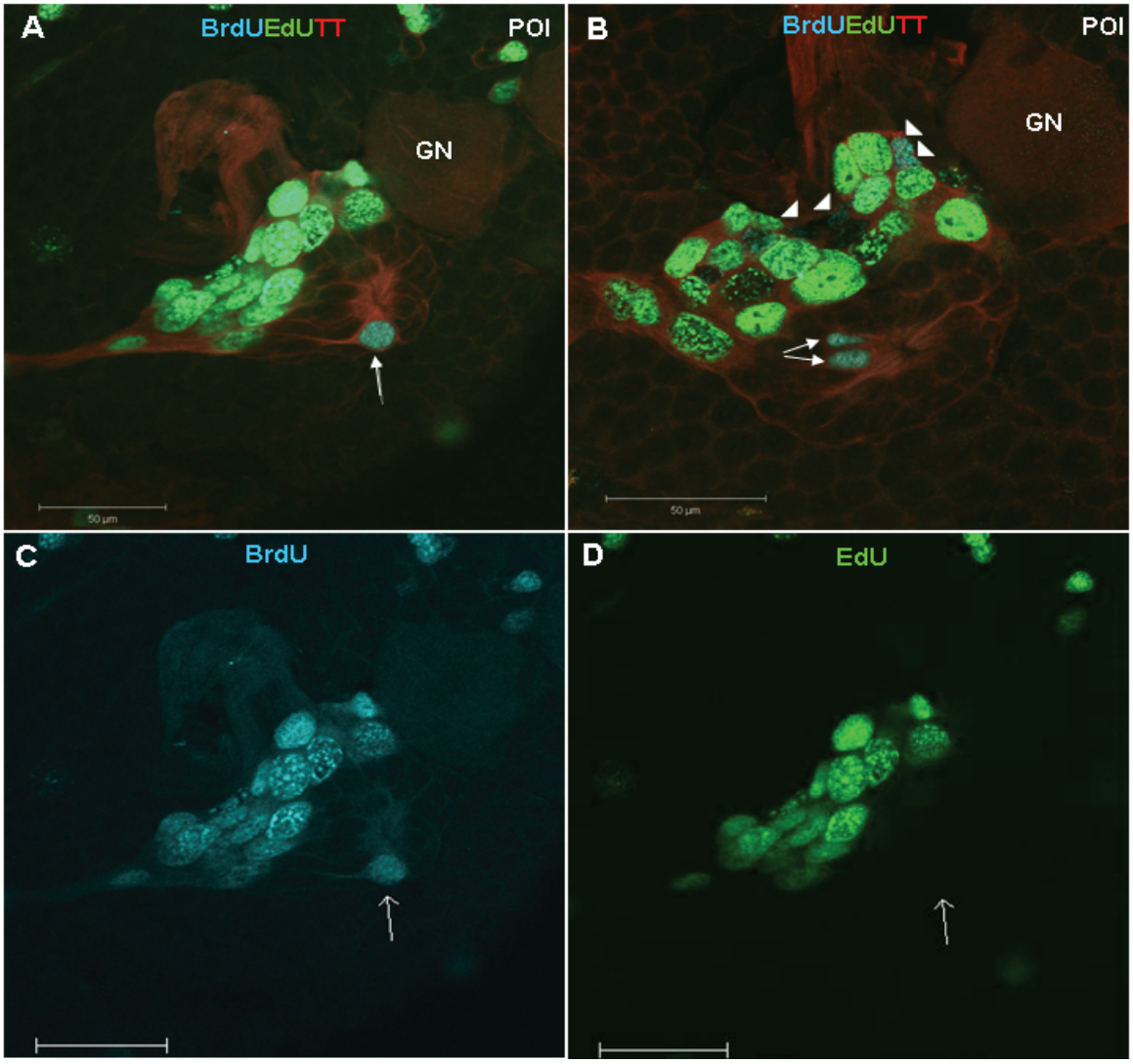


Plate 5.14- Pulse-chase experiment with BrdU (first pulse, 4 hours) and EdU (second pulse, 4 hours), 48 hours interval in between. POI stage. Triple staining with BrdU, EdU and Tyrosinated Tubulin (TT). A: the BrdU progeny (white arrow) born 48 hours earlier by the cap cells (EdU+) moved inside the niche-core after the birth. B: some BrdU new born cells (white arrow-head) lye on the cap. C, D: blue (BrdU) and green (EdU) channel of the overview in A. Abbr: GN, giant neuron. Scale bar (C, D): 50µm.

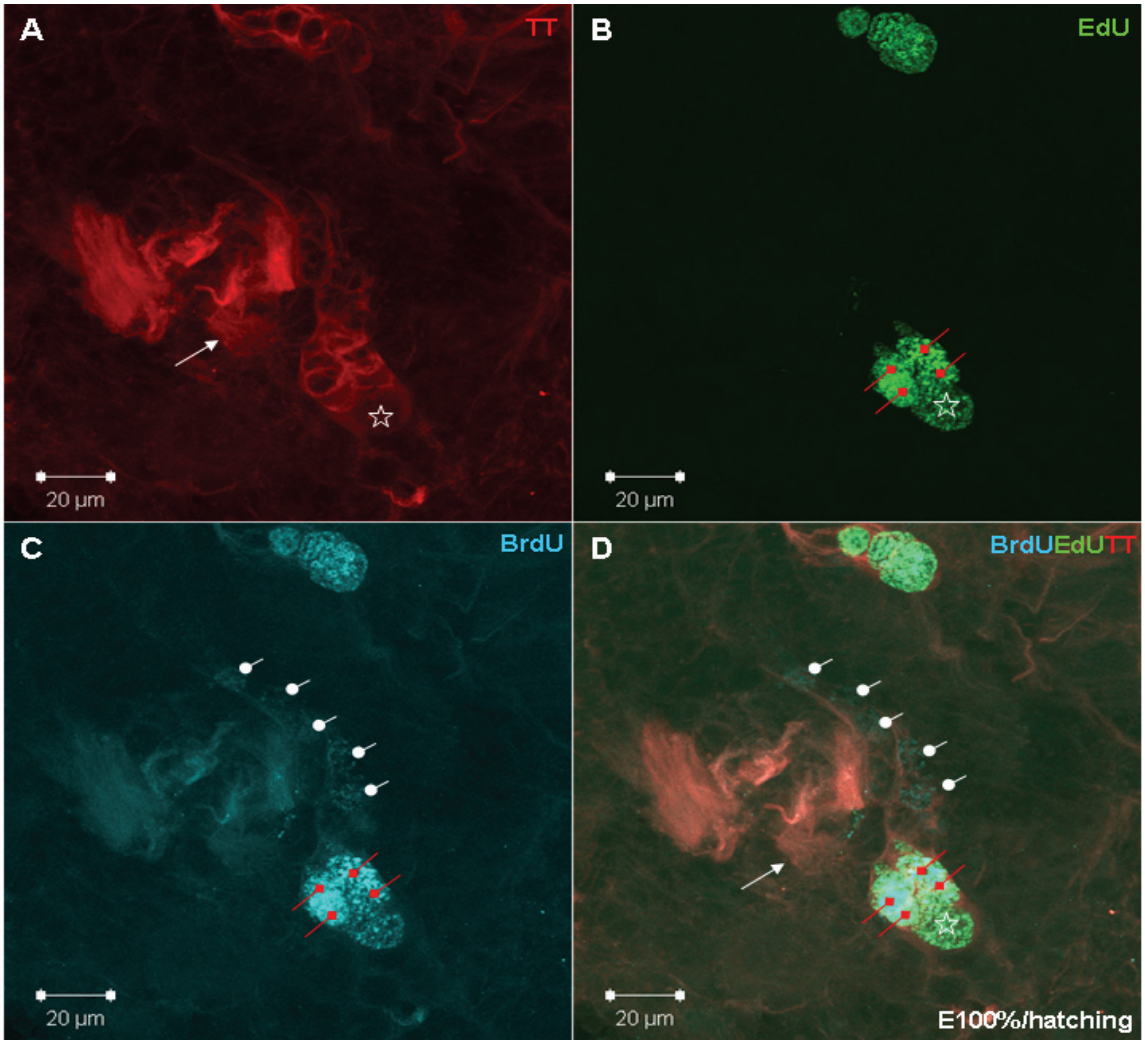


Plate 5.15- Pulse-chase experiment with BrdU (first pulse, 4 hours) and EdU (second pulse, 4 hours), 48 hours interval in between. End of embryogenesis (E100%/hatching). Triple staining with BrdU, EdU and Tyrosinated Tubulin (TT). A-C: separate color channels, the overview of the protoniche is shown in D. A: the proto-niche shows only the first outlines of the initial bunch of filaments (white arrow). B: the single mitotically active cell (white star) is labeled by EdU and, in C, by BrdU. The cell (neuroblast X) proliferates by giving off new born cells that either remain in place (red round-head) (B, C, D), or they are sent off along a pathway (white round-head) (C, D).

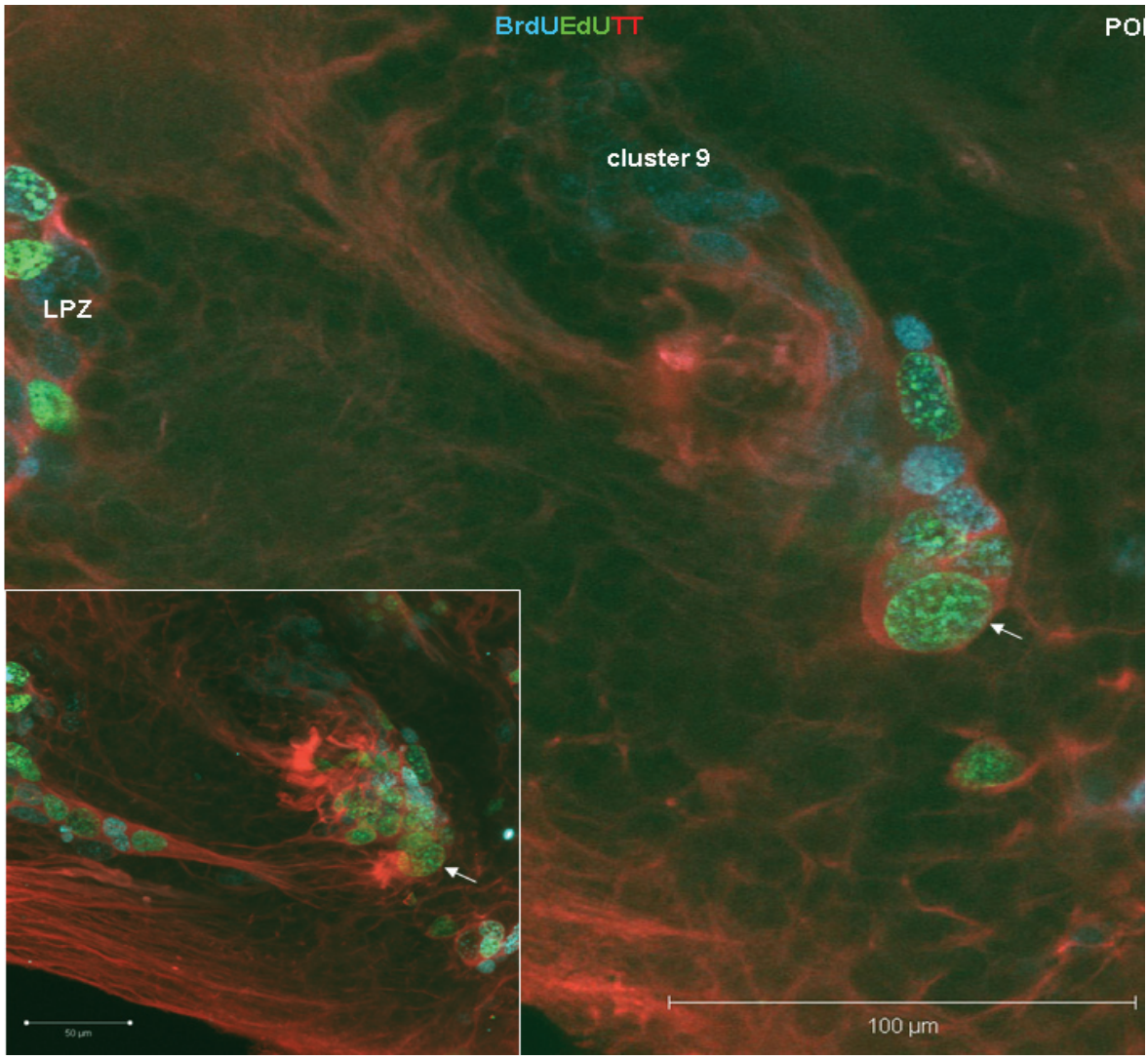


Plate 5.16- Pulse-chase experiment with BrdU (first pulse, 4 hours) and EdU (second pulse, 4 hours), 48 hours interval in between. Late POI stage. Triple staining with BrdU, EdU and Tyrosinated Tubulin (TT). Overview of the neuroblast X (white arrow), adjacent to the proto-niche (inset). Neuroblast X (EdU+, in green), together with cap cells (visible in the inset), contribute to the production of new born cells towards cluster 9. Abbr: LPZ, lateral proliferative zone.

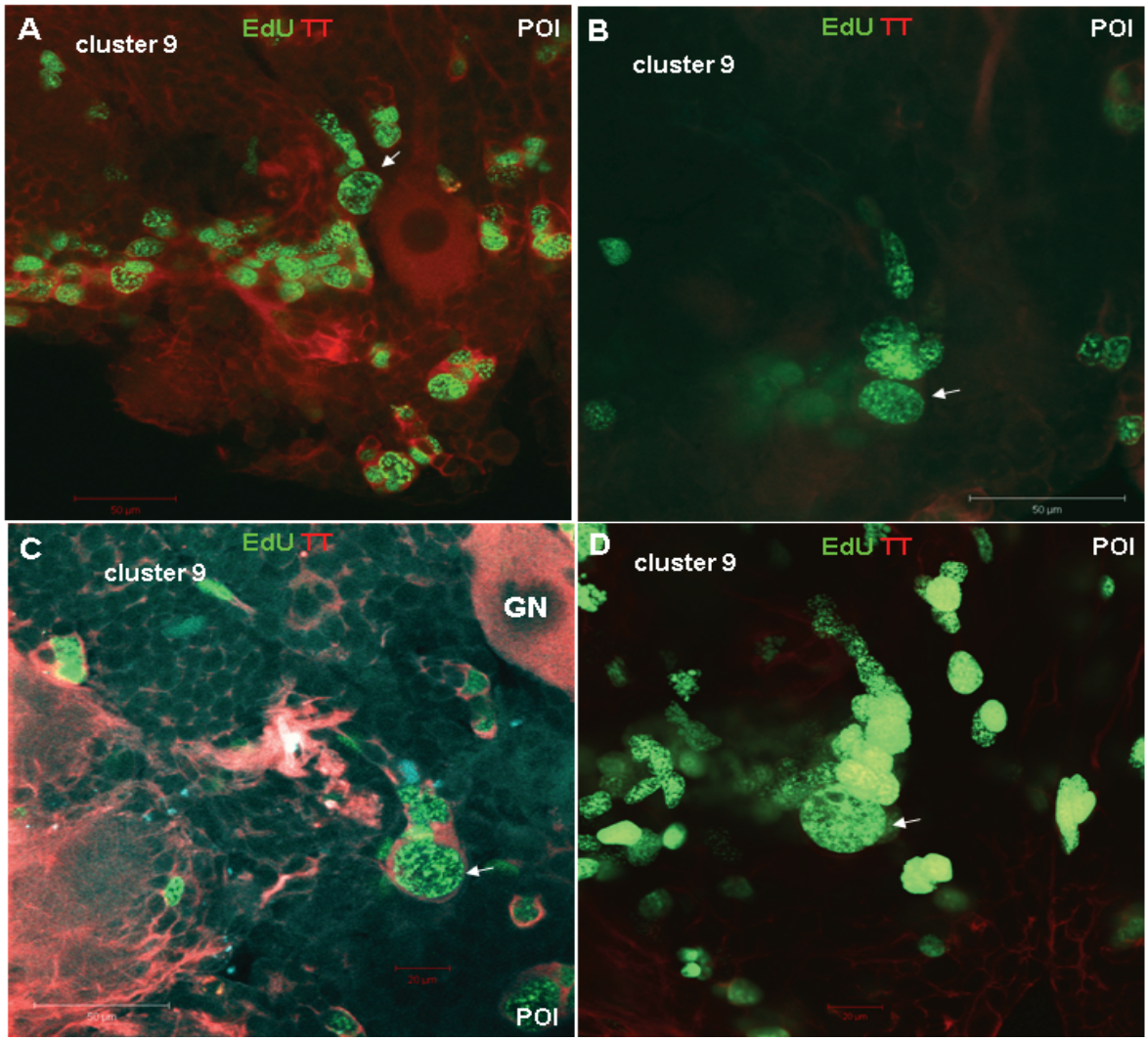


Plate 5.17- A: overview of the proto-niche and the neuroblast X (white arrow) during POI stage. B-D: Neuroblast X (white arrow). Double staining with EdU and Tyrosinated Tubulin (TT). Abbr: GN, giant neuron.

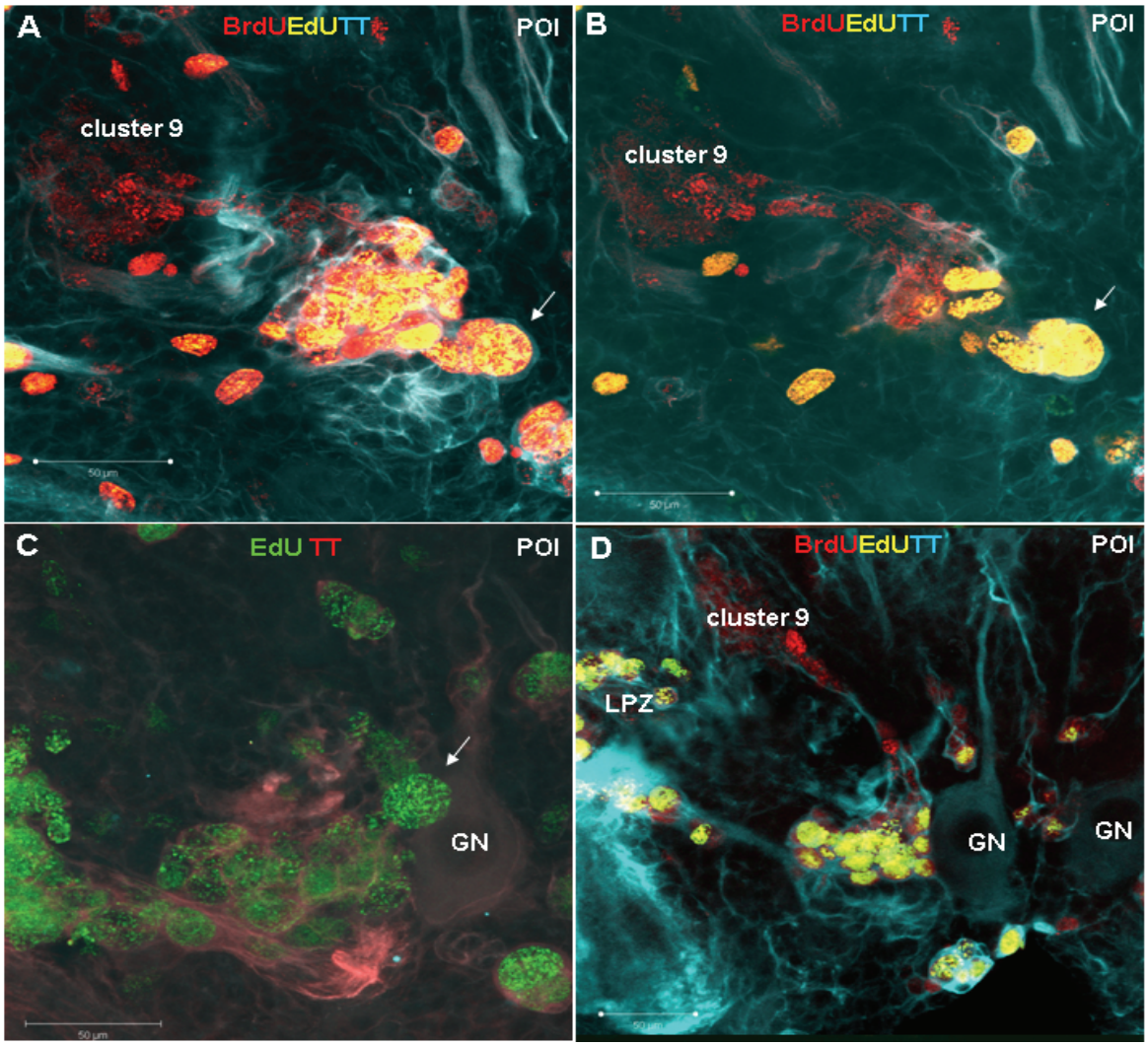


Plate 5.18- A, B, D: Pulse-chase experiment with BrdU (first pulse, 4 hours) and EdU (second pulse, 4 hours), 48 hours interval in between. Late POI stage. Triple staining with BrdU, EdU and Tyrosinated Tubulin (TT). C: Double staining with EdU and TT. A-C: neuroblast X (white arrow), adjacent to the proto-niche, contributes to cluster 9. Newborn cells migrate along the stream connecting the proto-niche to cluster 9 (A, B, D). Abbr: GN, giant neuron.

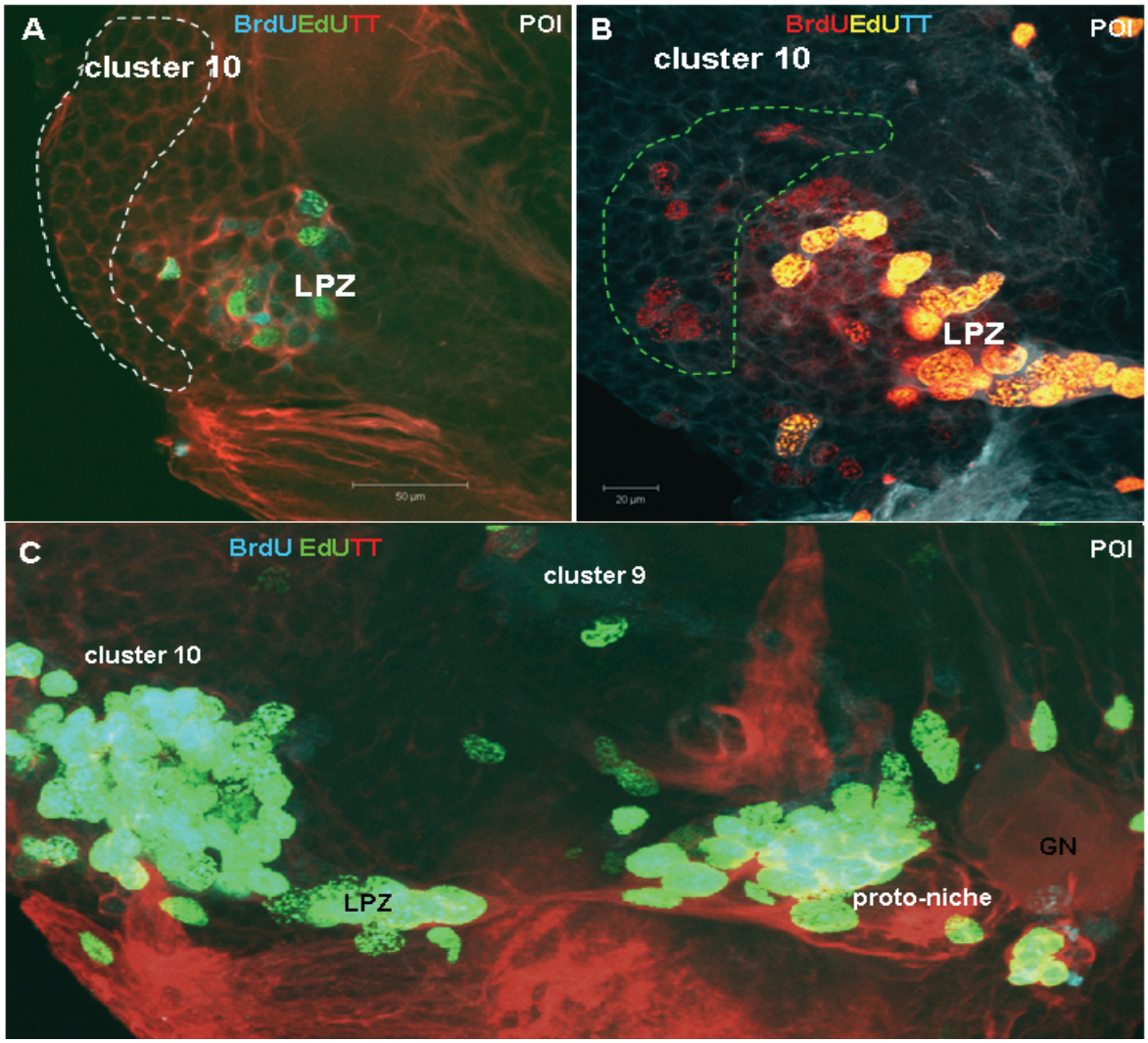


Plate 5.19- Pulse-chase experiment with BrdU (first pulse, 4 hours) and EdU (second pulse, 4 hours), 48 hours interval in between. Late POI stage. Triple staining with BrdU, EdU and Tyrosinated Tubulin (TT). A: cluster 10 (dotted line) and LPZ are shown. B: cluster 10, LPZ and the Transit Zone (green line) are shown. C: overview of the Deutocerebral Proliferative System. Abbr: LPZ, lateral proliferative zone, GN, giant neuron.

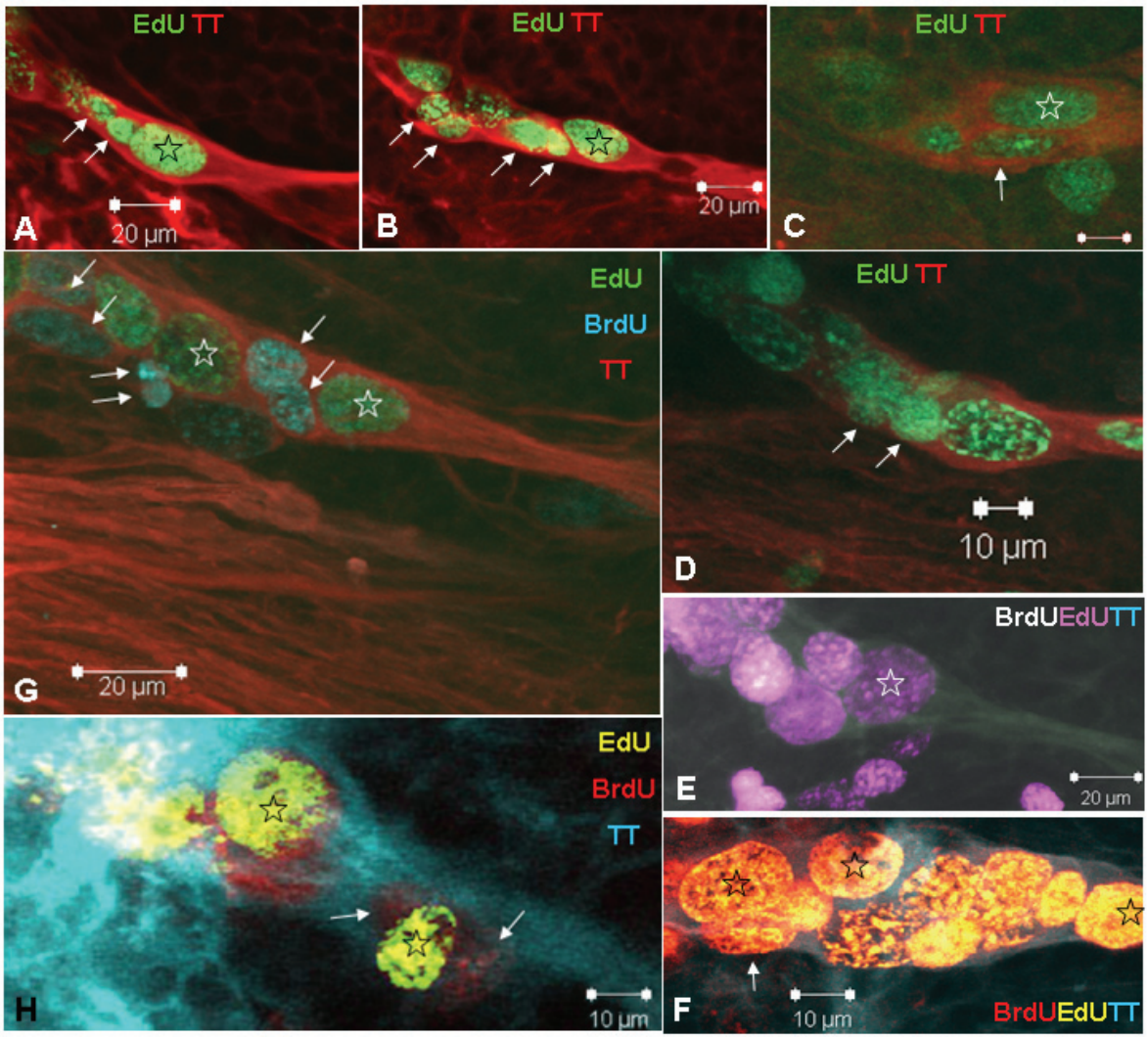


Plate 5.20- Higher magnifications of the LPZ. Late POI stage. A-D: double staining with EdU and Tyrosinated Tubulin (TT). E-H: pulse-chase experiment with BrdU (first pulse, 4 hours) and EdU (second pulse, 4 hours), 48 hours interval in between. Two or three bigger cells (white or black star) are within zone and associated to smaller progeny (white arrow).

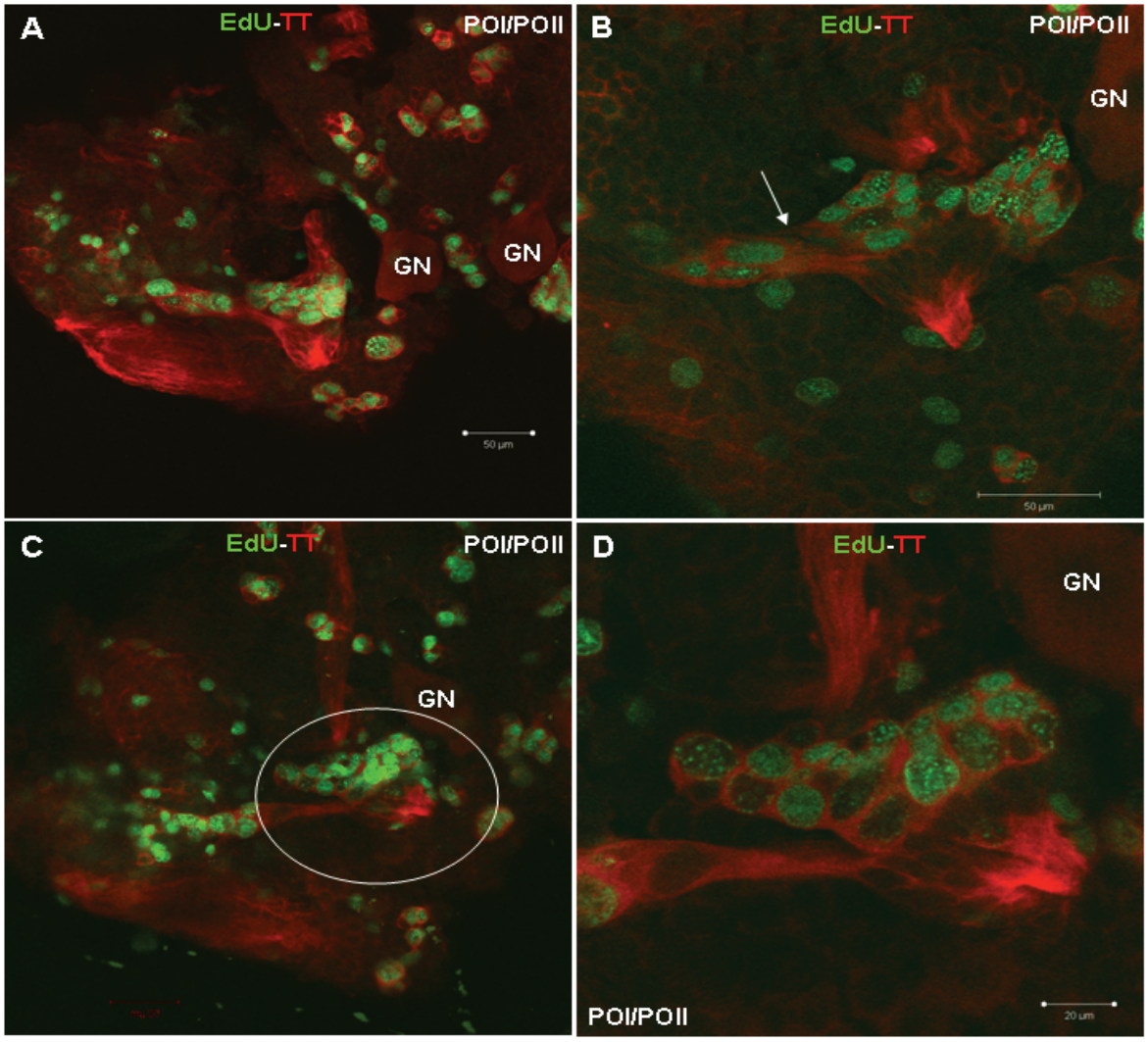


Plate 5.21- Detachment of the cap from the niche-core, between POI and POII stage. Double staining with EdU and Tyrosinated Tubulin (TT). A: note that some NBs are still patchily disperded in the brain. B: detachment of the cap (white arrow). C: overview of the detachment of the cap (white circle) that is magnified in D. Abbr: GN, giant neuron.

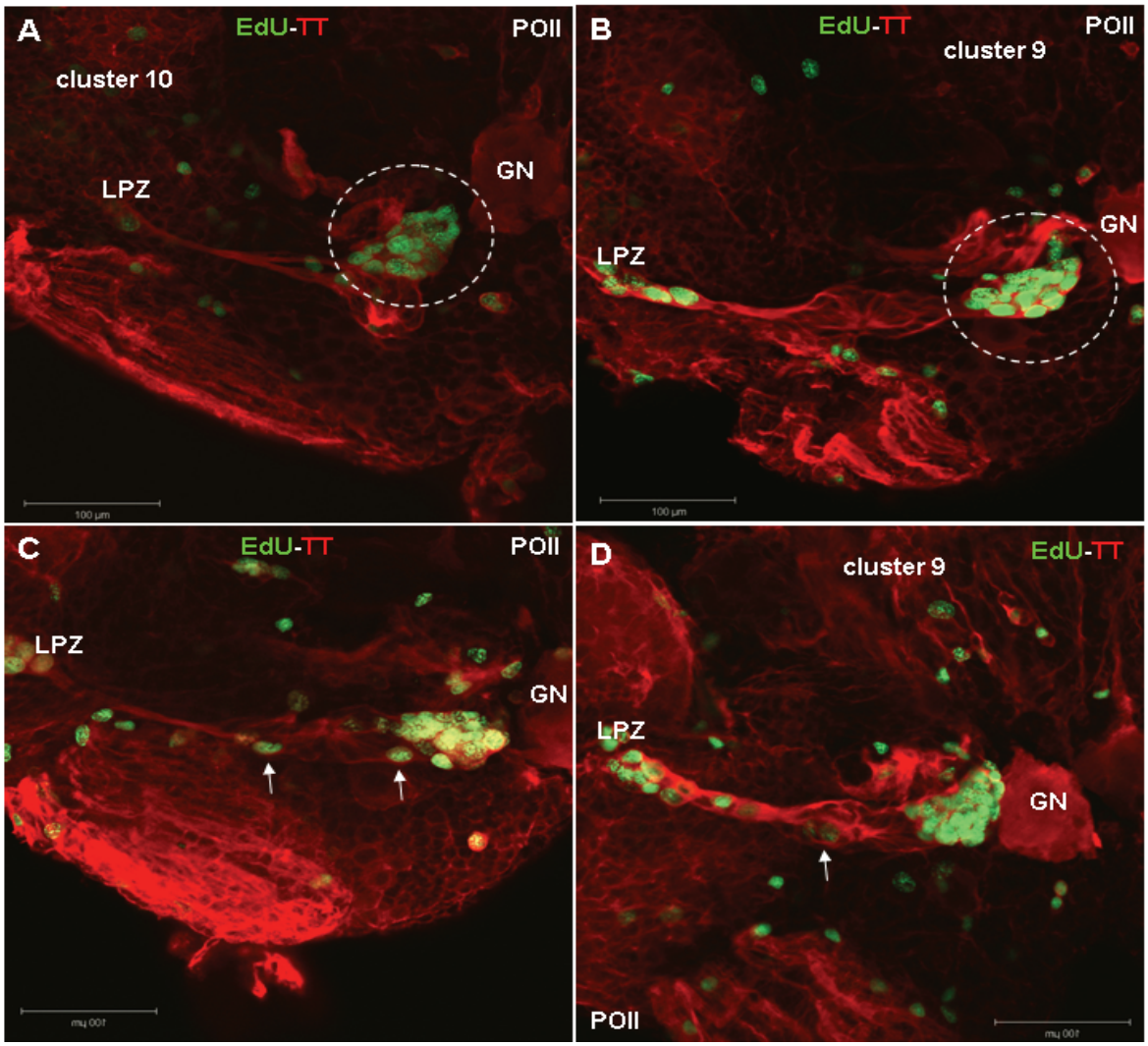


Plate 5.22- Displacement of the cap towards cluster 9. POII stage. Double staining with EdU and Tyrosinated Tubulin (TT). A-B: the cap (dotted circle) gets displaced from the niche-core that from now on is called "niche". The niche is between the cap and LPZ. C, D: EdU+ cells (arrow) appear within the niche, at its lateral endings, almost symmetrically. Abbr: GN, giant neuron, X, Neuroblast X, LPZ, lateral proliferative zone.

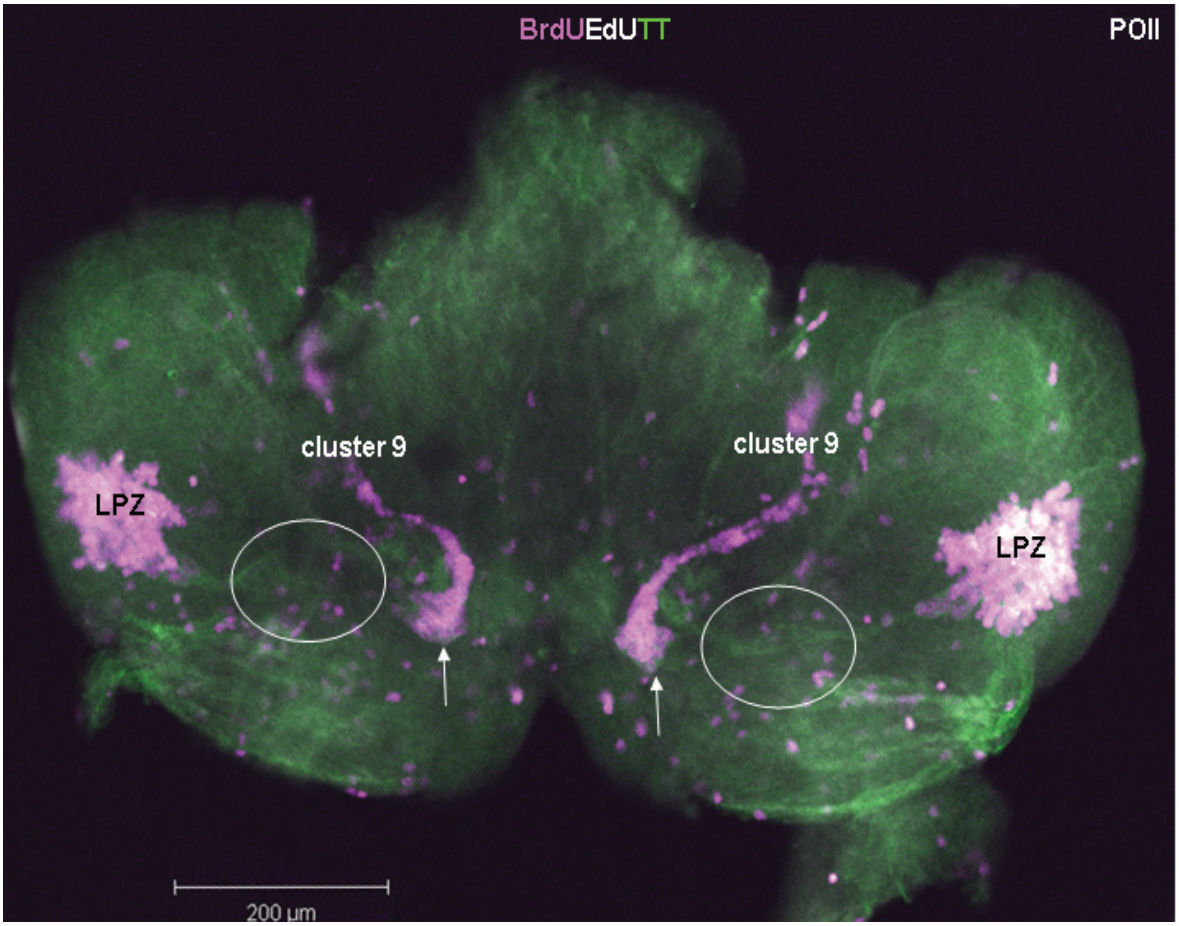


Plate 5.23- Pulse-chase experiment with BrdU (first pulse, 4 hours) and EdU (second pulse, 4 hours), 48 hours interval in between. POII stage. Triple staining with BrdU, EdU and Tyrosinated Tubulin (TT). Displacement of the cap (white arrow) towards cluster 9. The niche (white circle) is between the cap and LPZ. Abbr: LPZ, lateral proliferative zone.

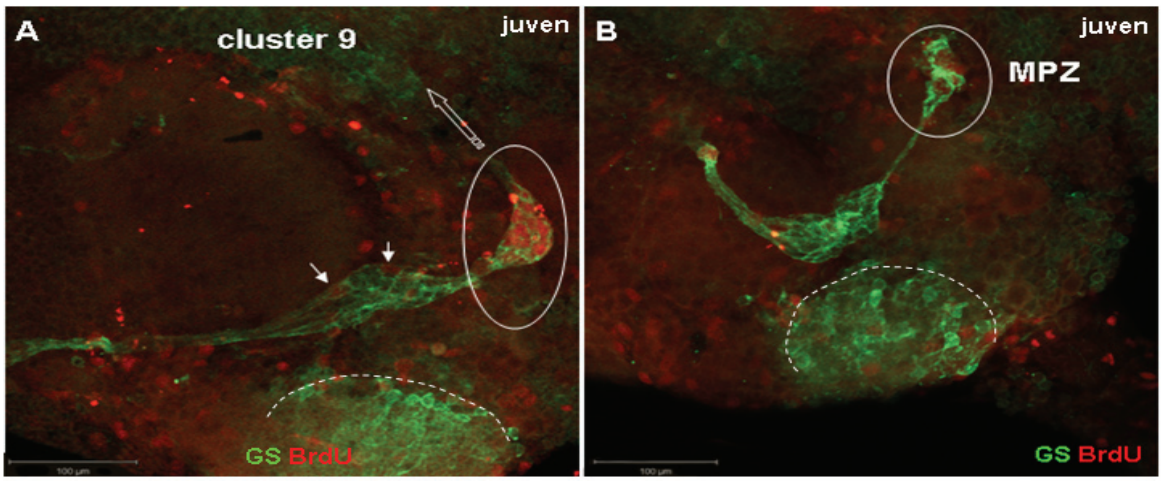


Plate 5.24- Displacement of the cap towards cluster 9. POII/early juveniles. Double staining with BrdU and Gluthamine synthetase (GS). A: in some specimens, the cap (white circle) appears just detached from the niche and towards (stripped white arrow) cluster 9. A couple of BrdU+ cells are visible within the niche (white arrow). B: in some specimens, the cap has already taken distance from the niche and reached cluster 9, by becoming MPZ (medial proliferative zone) (white circle). A group of GS+ cells is visible in proximity of the niche (dotted line) (A, B).

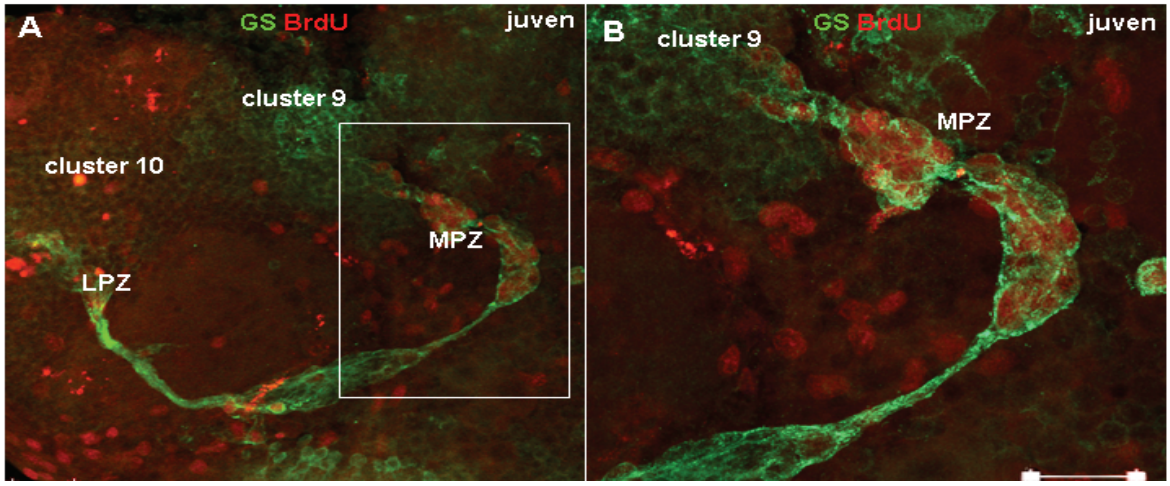


Plate 5.25- The niche and the Medial Proliferative Zone (MPZ). Early juveniles. Double staining with BrdU and Gluthamine synthetase (GS). A: the cap has become the MPZ, BrdU+ cells look packed and wrapped by filaments within the MPZ. B: higher magnification of the MPZ in A. Abbr: LPZ, lateral proliferative zone.

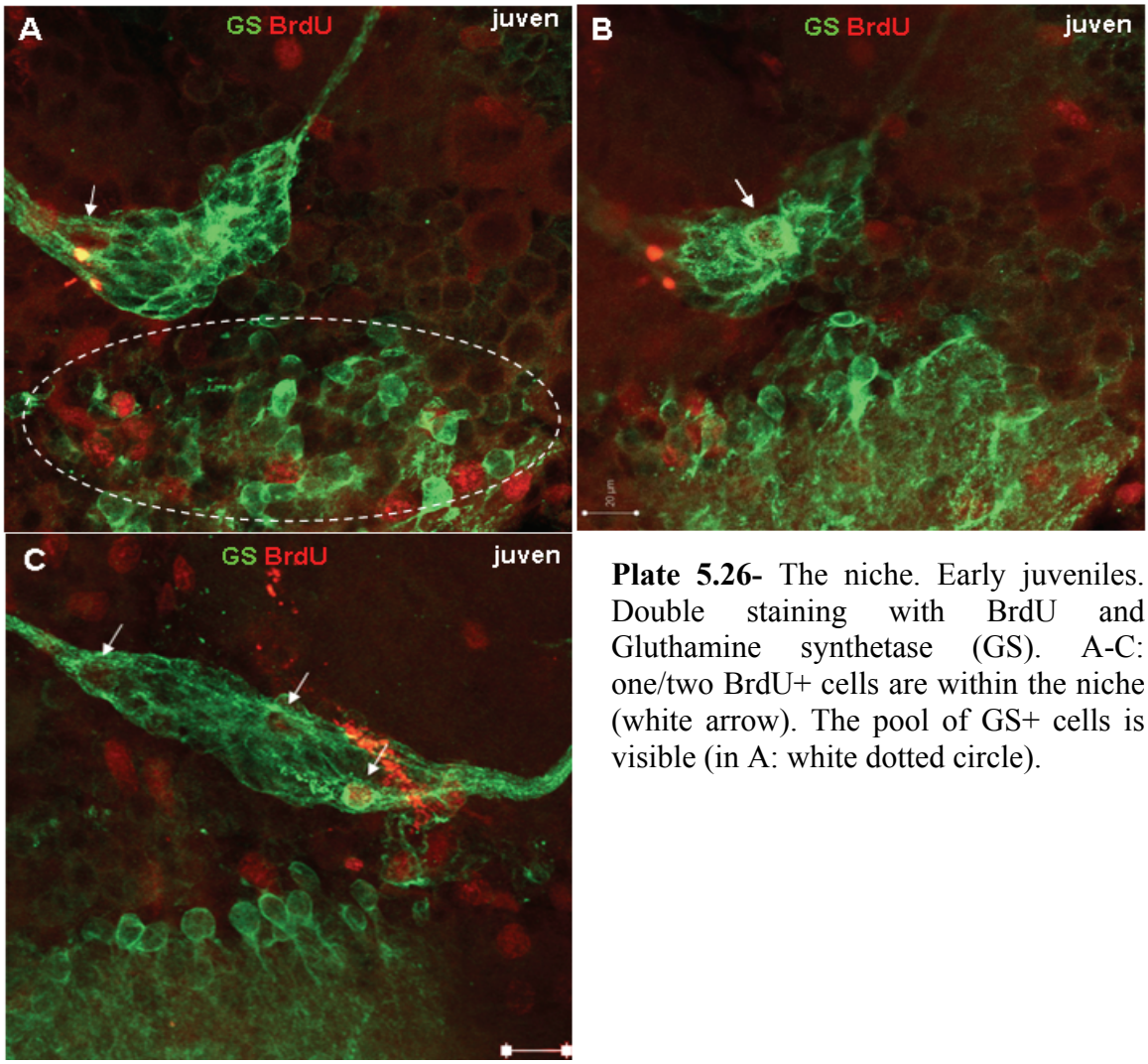


Plate 5.26- The niche. Early juveniles. Double staining with BrdU and Gluthamine synthetase (GS). A-C: one/two BrdU+ cells are within the niche (white arrow). The pool of GS+ cells is visible (in A: white dotted circle).

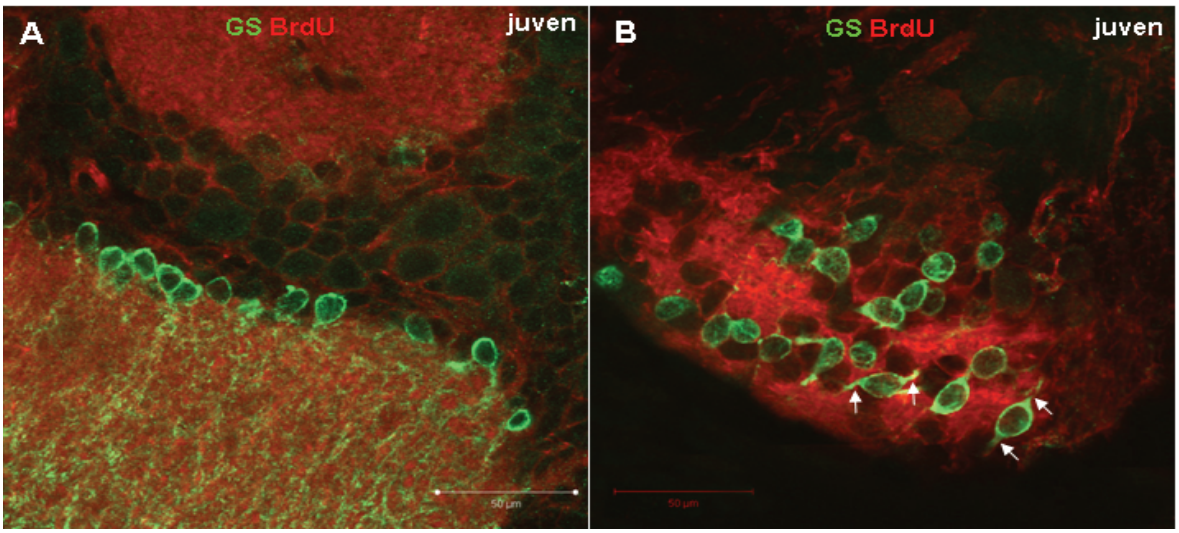


Plate 5.27- Higher magnification of the gluthamine synthetase (GS)+ cell pool. Double staining with BrdU and GS. A, B: cells are spherical with two lateral cytoplasmatic extensions (white arrow).

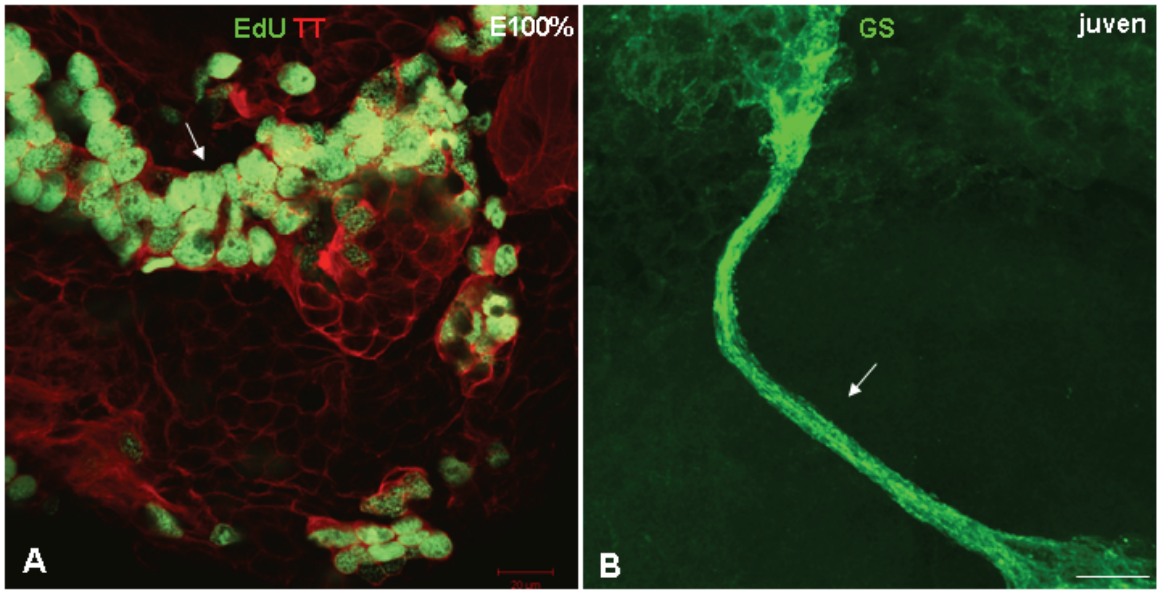


Plate 5.28- Comparison of the migratory stream (arrow) between the end of embryogenesis stage (E100%) and the juveniles stage. A: E100%. Double staining with EdU and Tyrosinated Tubulin (TT). The stream (white arrow) is a large band of aggregated EdU+ cells. B: juveniles stage. Gluthamine synthetase (GS) staining. The stream has shrunken in size and looks like a thin string connecting the niche to the two proliferative zones. Scale bar: 50µm.

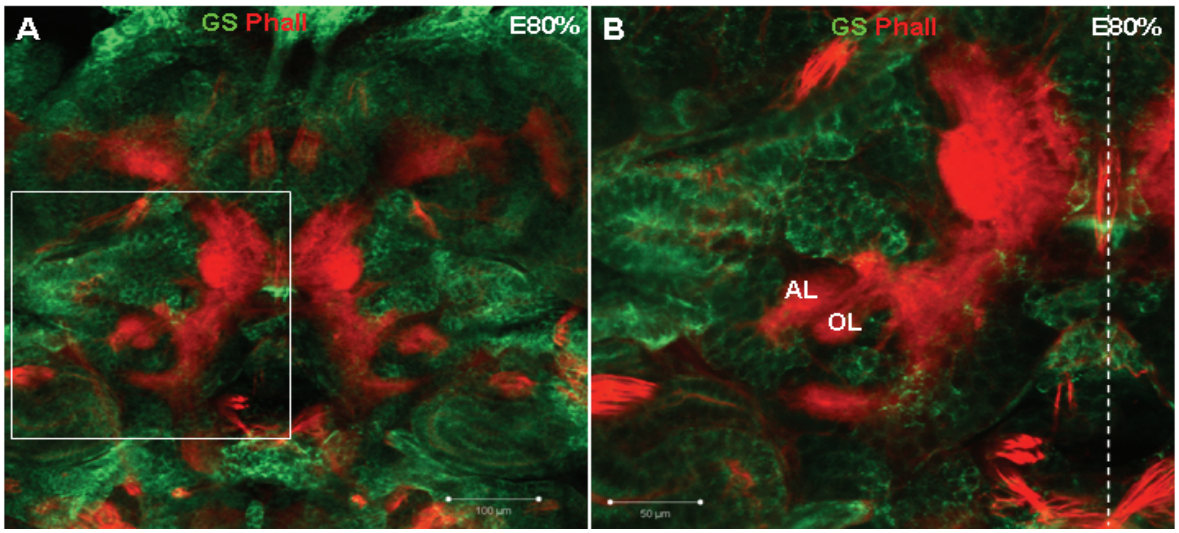


Plate 5.29- A: overview of the brain at E80%. Double staining with Phalloidin (Phall) and Gluthamine synthetase (GS). B: higher magnification of the deutocerebrum (white square in A): olfactory (OL) and accessory lobes (AL) emerge in the deutocerebrum. Dotted line: midline.

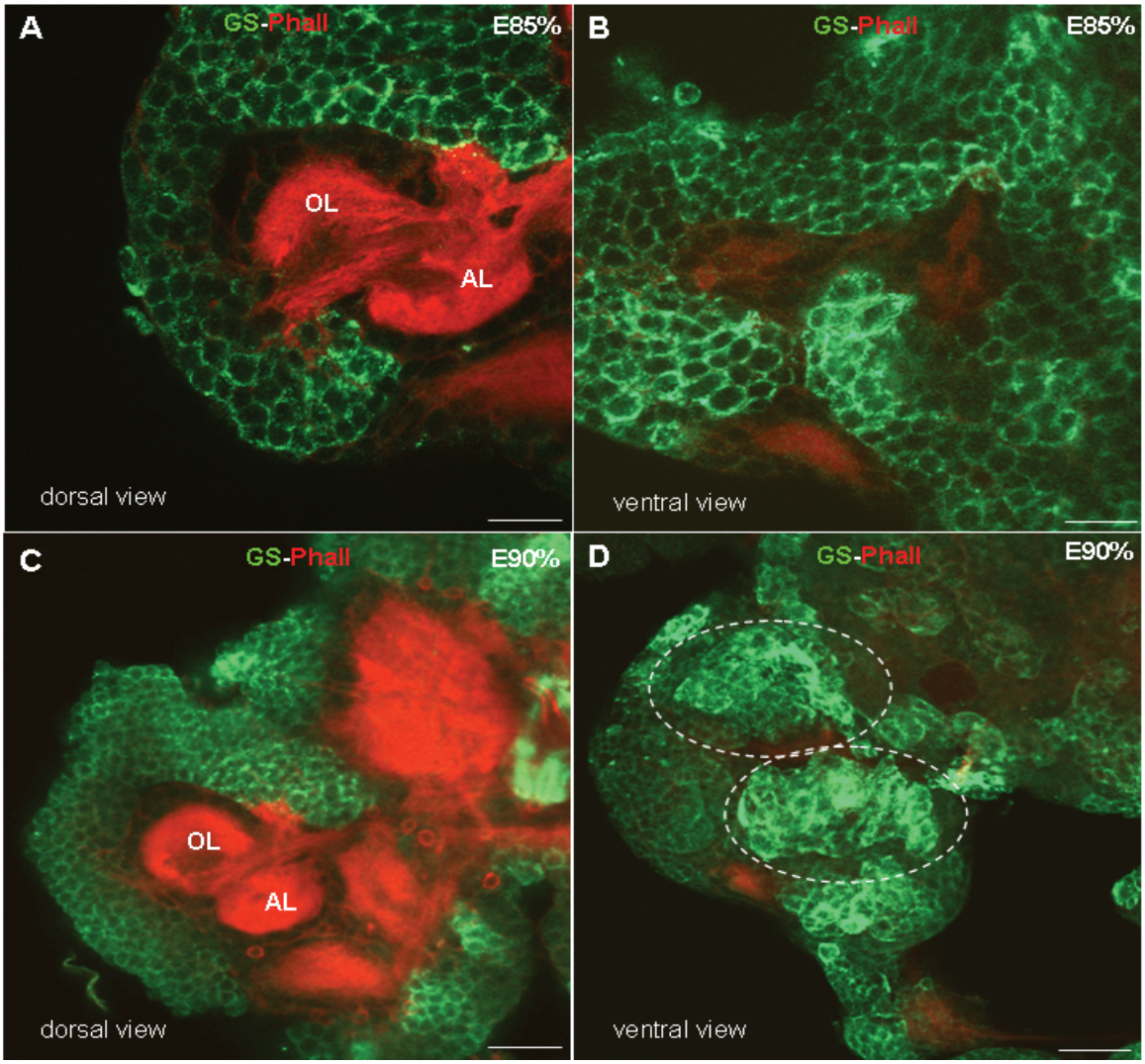


Plate 5.30- Dorsal (A, C) and ventral (B, D) overviews of the deutocerebrum during embryogenesis. Double staining with Phalloidin (Phall) and Gluthamine synthetase (GS). A: E85%. Dorsal view, olfactory (OL) and accessory lobes (AL) are forming. B: E85%. ventral view, GS+ cells are uniformly dispersed on the surface. C: E90%. Dorsal view, olfactory (OL) and accessory lobes (AL) are forming. D: E90%. Ventral view, GS+ cells aggregate in more compact groups of cells (dotted white circle). Scale bar: 100µm.

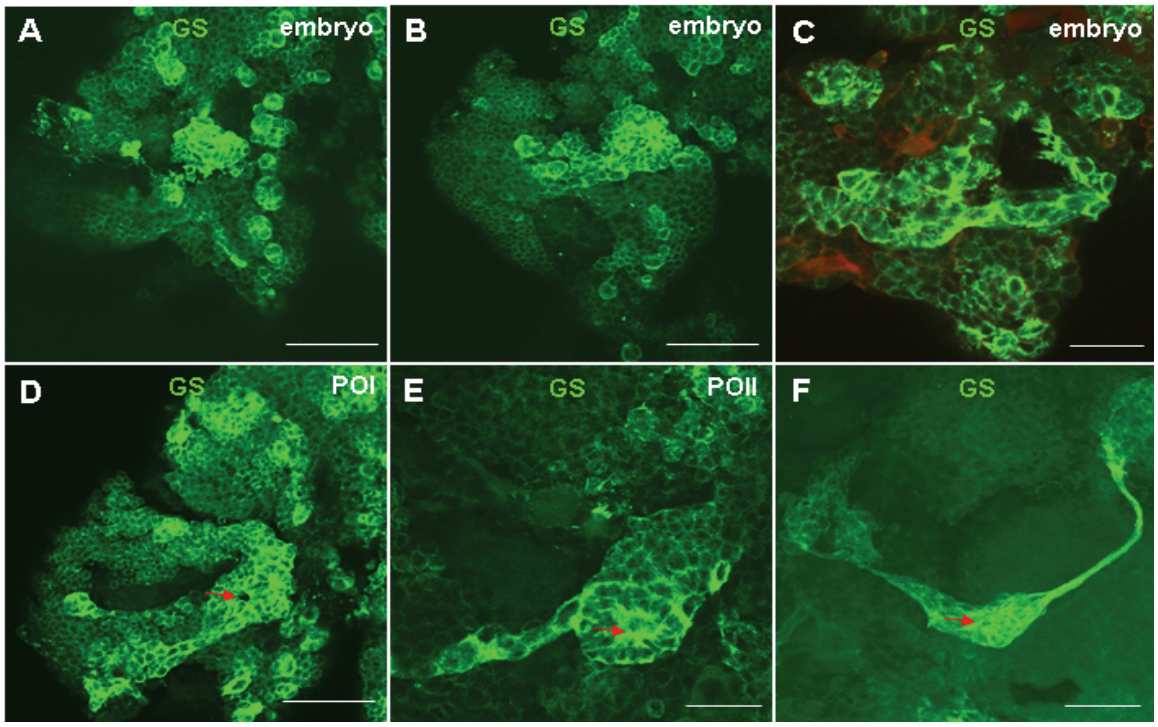


Plate 5.31- Development of the niche on the ventral surface of the brain of the Marbled crayfish, stained with gluthamine synthetase (GS) (A-F). A –C: the group of GS+ cells gets aggregated and takes shape during the final stages of embryogenesis. D: during POI the central pore becomes visible (red arrow). E: in POII, the cap detaches from the niche-core towards cluster 9. F: in juvenile the niche has achieved its mature structure. Scale bar: 50µm.

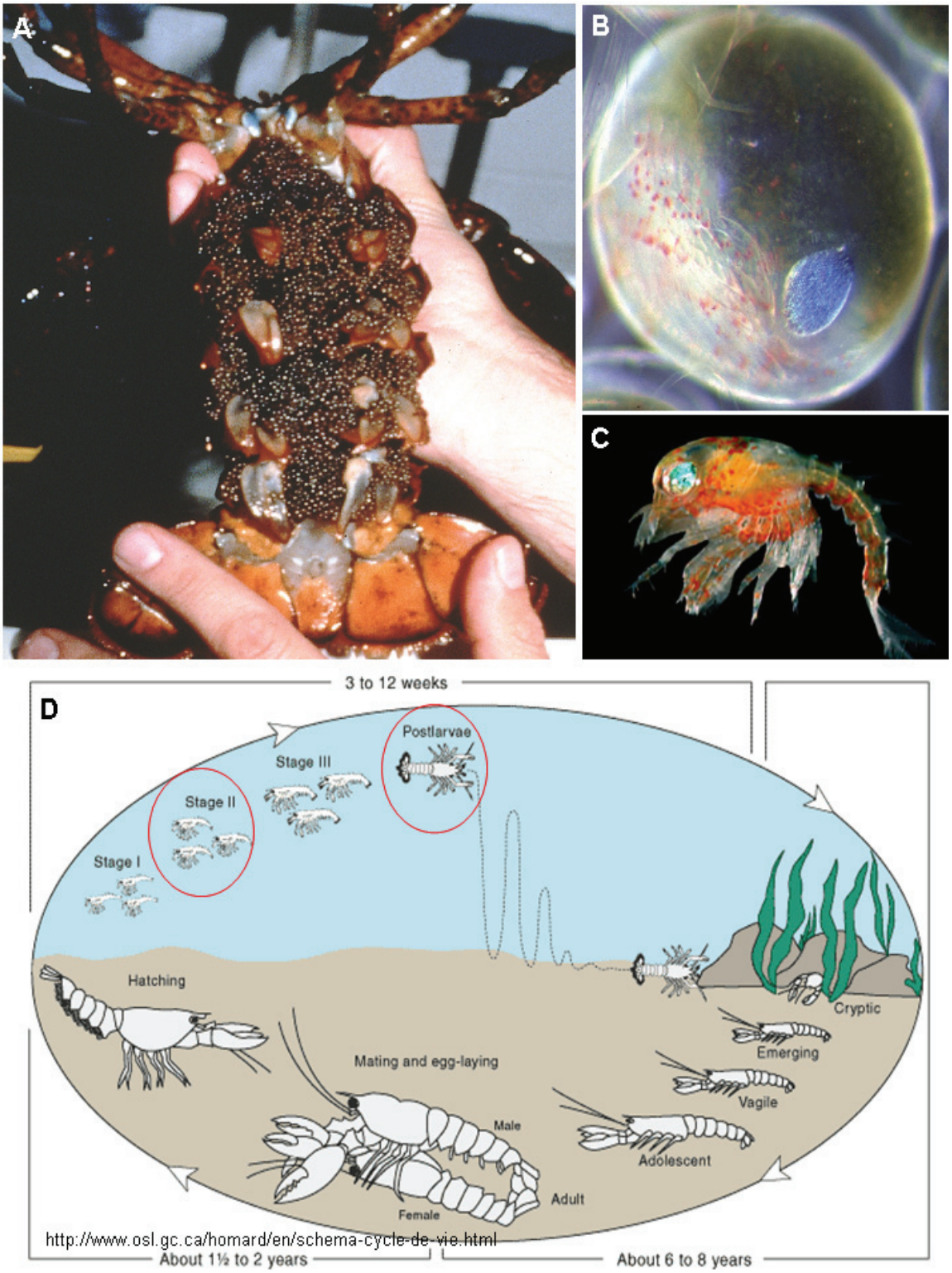


Plate 5.32- The American lobster (*Homarus americanus*) and its life cycle. A: the female lobster bearing eggs. B: higher magnification of the embryo within the egg. C: the lobster larva. D: the American lobster life cycle. When eggs hatch, lobsters go through four larval stages characterized by one molt in between. Larvae IV (postlarvae) resemble the adult shape and by this time the animal is about one month old. This stage may last several weeks. Stages II and IV (red circle) are the life stages I mostly used for the current project.

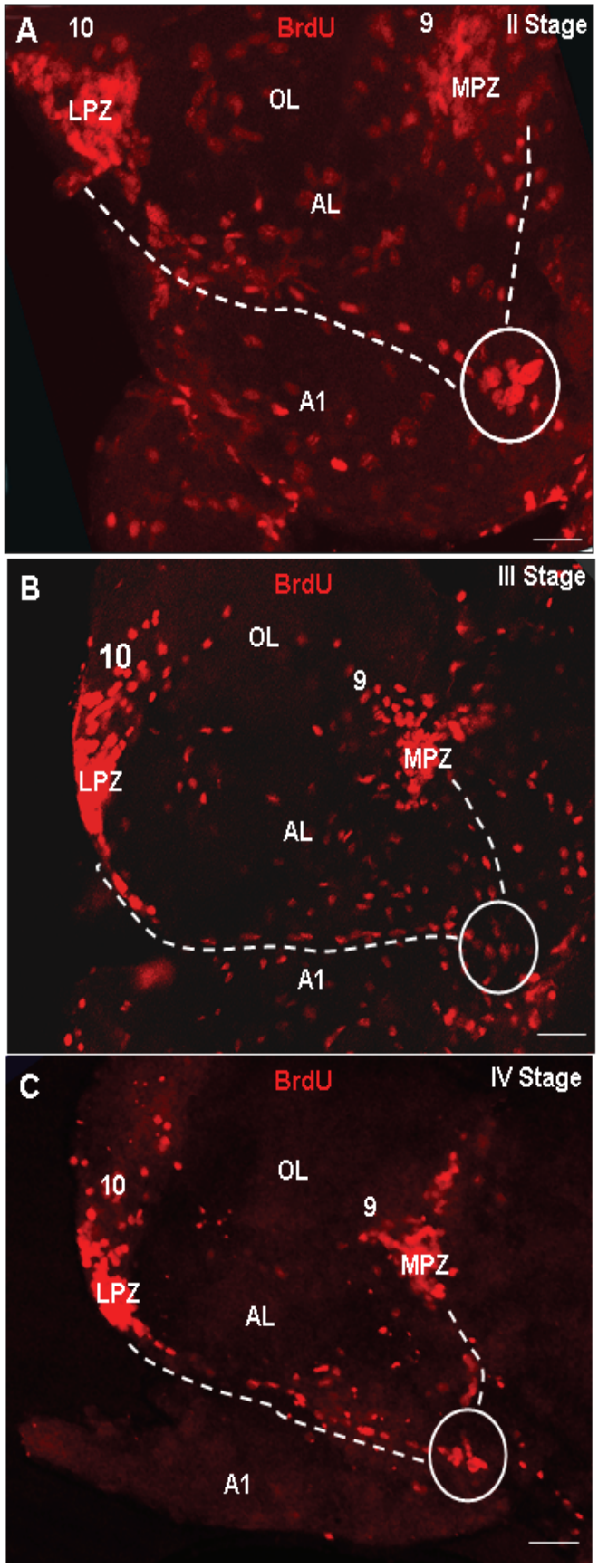


Plate 5.33- Overview of the deutocerebrum at different larval stages of the American lobster (*Homarus americanus*). BrdU staining. A: II stage. B: III stage. C: IV stage. A-C: the two lateral proliferative zones (LPZ and MPZ) are visible, two streams of cells (dotted line) converge to one point in between (white circle). Abbr: OL, olfactory lobe, AL, accessory lobe, LPZ, lateral proliferative zone, MPZ, medial proliferative zone, 10, cluster 10, 9, cluster 9, A1, antenna 1. Scale bar: 50µm.

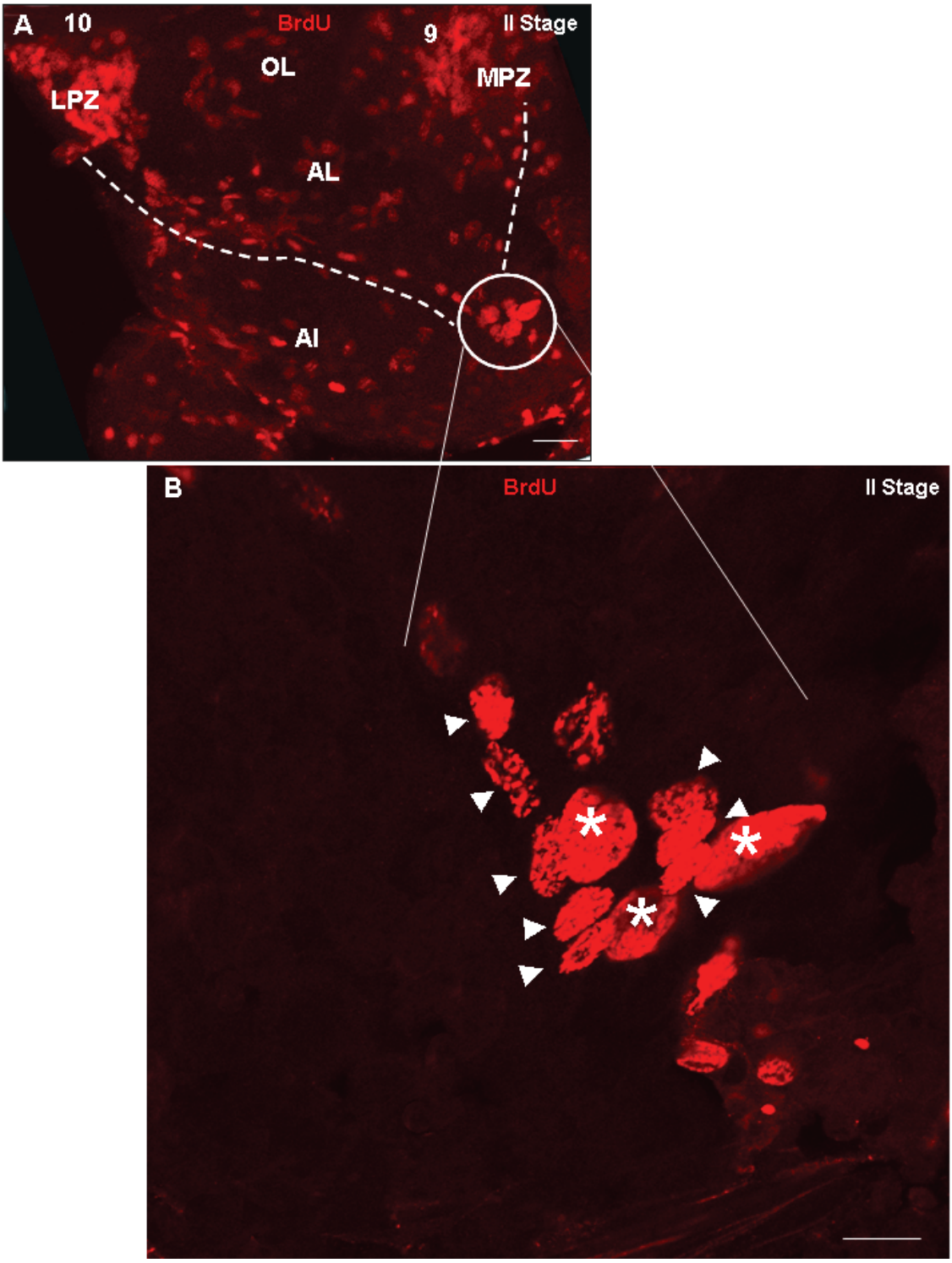


Plate 5.34- Overview of the deutocerebrum in II stage larvae of the American lobster (*Homarus americanus*). BrdU staining. A: overview. LPZ and MPZ are joint to a small group of cell (circle) by two streams of cells (dotted line). B: higher magnification of the group of cells (circle in A) placed in between LPZ and MPZ. Three bigger cells (white asterisk) are among smaller cells (white arrowhead). Abbr: OL, olfactory lobe, AL, accessory lobe, LPZ, lateral proliferative zone, MPZ, medial proliferative zone, 10, cluster 10, 9, cluster 9, A1, antenna 1. Scale bar: 50µm.

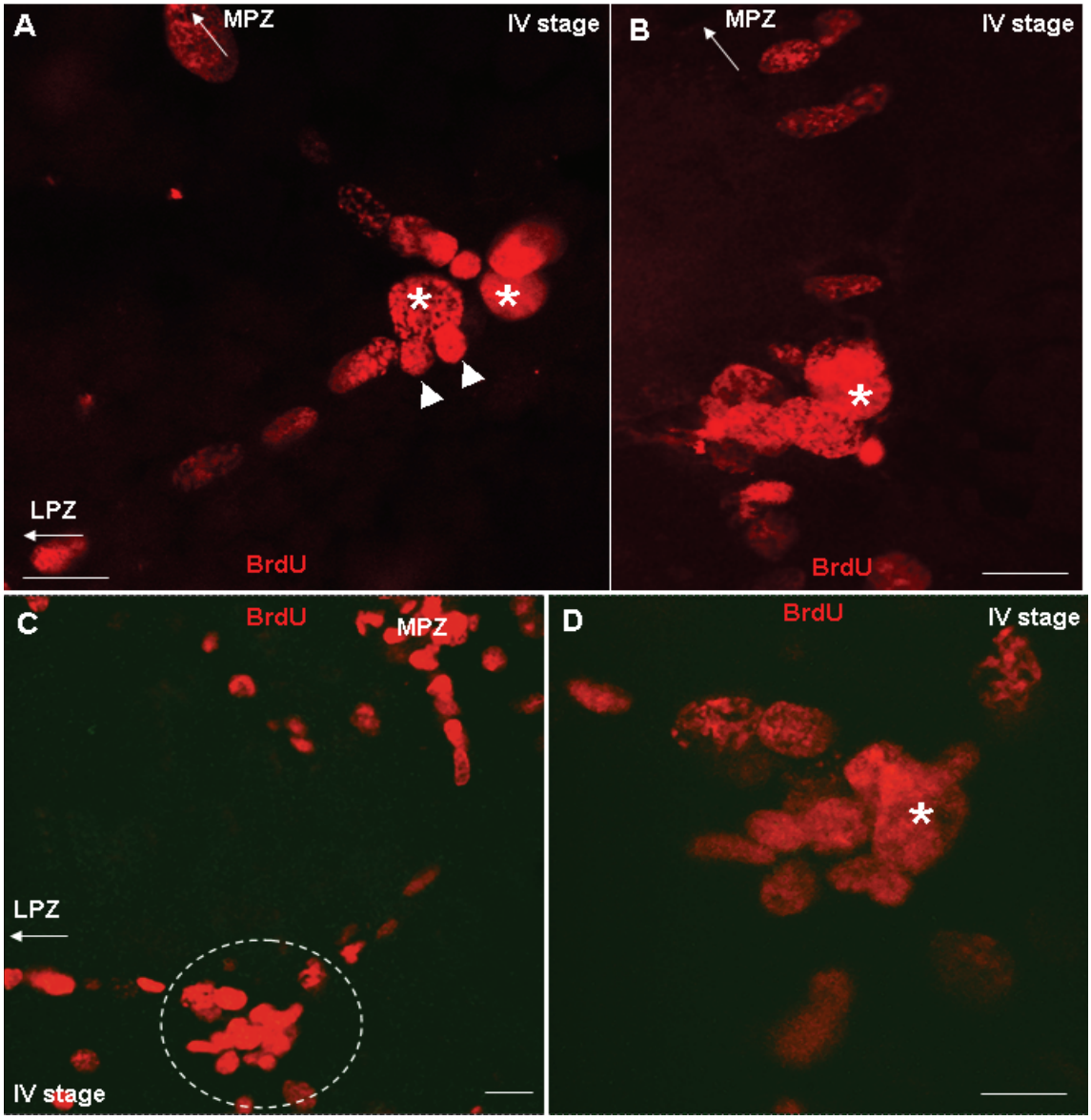


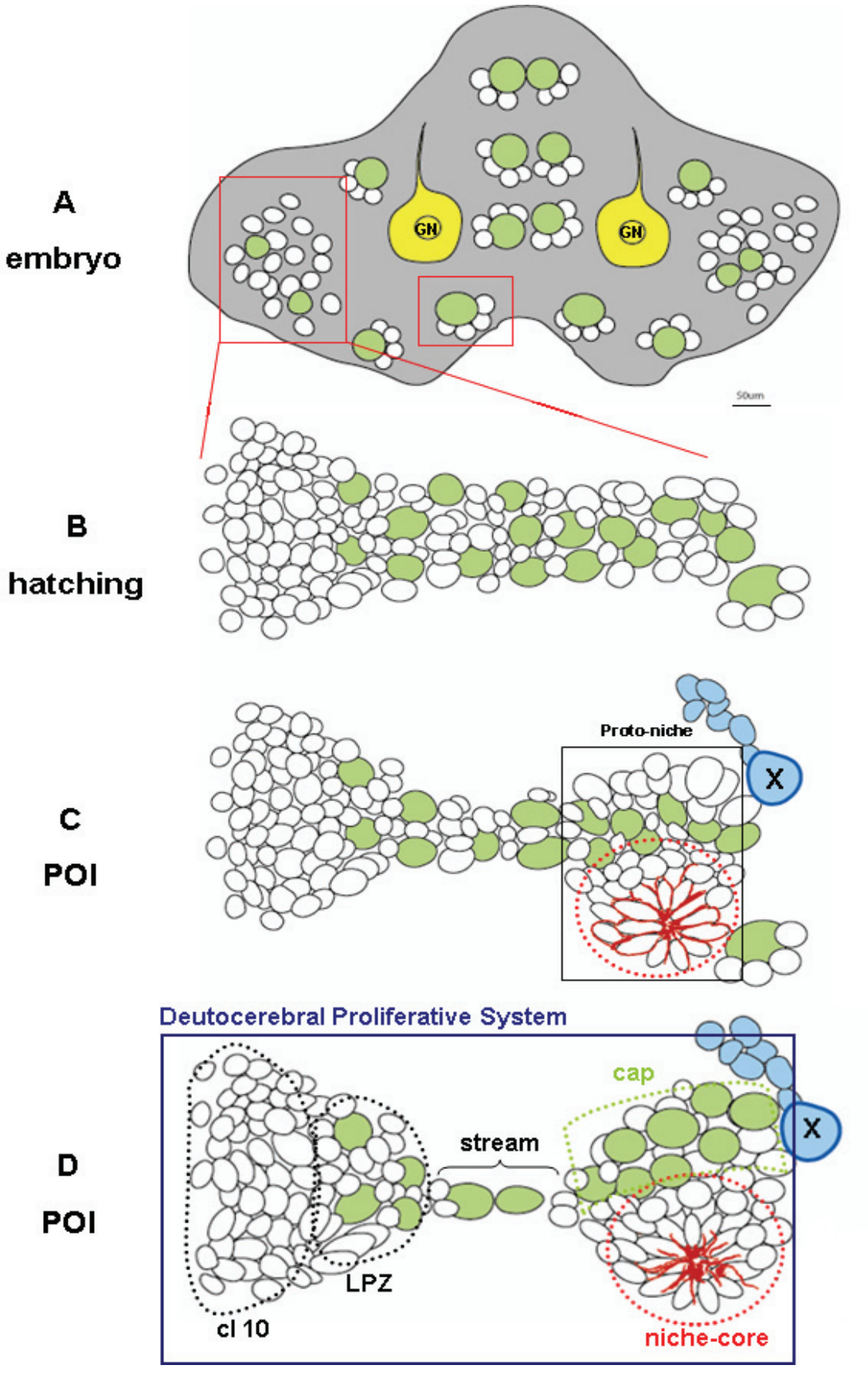
Plate 5.35- Proliferative group of cells in IV stage larvae of the American lobster (*Homarus americanus*). BrdU staining. A, B: few bigger cells (asterisks) are associated to smaller cells. Some of them have been just budded off (white arrowhead) C: overview of the proliferative group of cells (dotted circle) placed in between the two streams. D: higher magnification of the proliferative group. One bigger cell (asterisks) is among smaller cells. Abbr: LPZ, lateral proliferative zone, MPZ, medial proliferative zone. Scale bar: 50µm.

5.3 DISCUSSION

5.3.1. A model for the development of the niche in the Marbled crayfish brain

At a specific time between end of embryogenesis and beginning of post-embryonic life, the neurogenic niche becomes visible on the ventral surface of the brain (Fig. 1A, B). It can be hypothesized that the emergence of the niche is orchestrated by a hormonal signal that activates the expression of specific genes and is part of the hormonal activity that coordinates the molting process during hatching. In insects, it has been shown that progenitor cell activity (such as neuroblasts) depends on genetic programs but also on hormonal signals, both during embryogenesis and adult life (Cayre et al. 1994, 2007. Strambi et al., 1999, Tissot and Stocker 2000, Harrison et al. 2000). For instance, the molting process in both insects and crustaceans is controlled by the so called "molting hormone", hormone 20-hydroxyecdysone, which causes the secretion of a new cuticle but it also essential for initiating the changes of the animal's internal morphology that are typical for the new stage (Harrison et al. 2000). Recent studies on the development of the ovary niche in *Drosophila* hypothesized that the hormone ecdysone could mediate such process (Zhu and Xie, 2003). It is also known that hormonal cycles influence the rate of neurogenesis in decapod crustaceans (Beltz and Sandeman 2003, Benton et al. 2008) and that neurotransmitters and growth factors are implicated in the regulation of adult neurogenesis in invertebrates. Experiments of serotonin depletion in crustaceans show that this neurotransmitter stimulates the proliferation of neuronal progenitors, as it does in mammals (Beltz et al. 2001, Benton and Beltz 2001). However, detailed studies on factors that trigger the formation of the newly discovered neurogenic niche in decapod crustaceans are not available so far, so that more experiments in this direction need to be done.

When the egg begins to break (hatching) and the animal enters the POI phase, a subset of cells on the ventral surface of each half of the brain takes shape by aggregating into a more compact cluster of cells (Fig. 1B, C). These cells organize around a bunch of filaments which simultaneously are forming and that probably derive from the cells themselves.


A
embryo
GN
GN
50um
B
hatching
C
POI
Proto-niche
X
Deutocerebral Proliferative System
D
POI
cap
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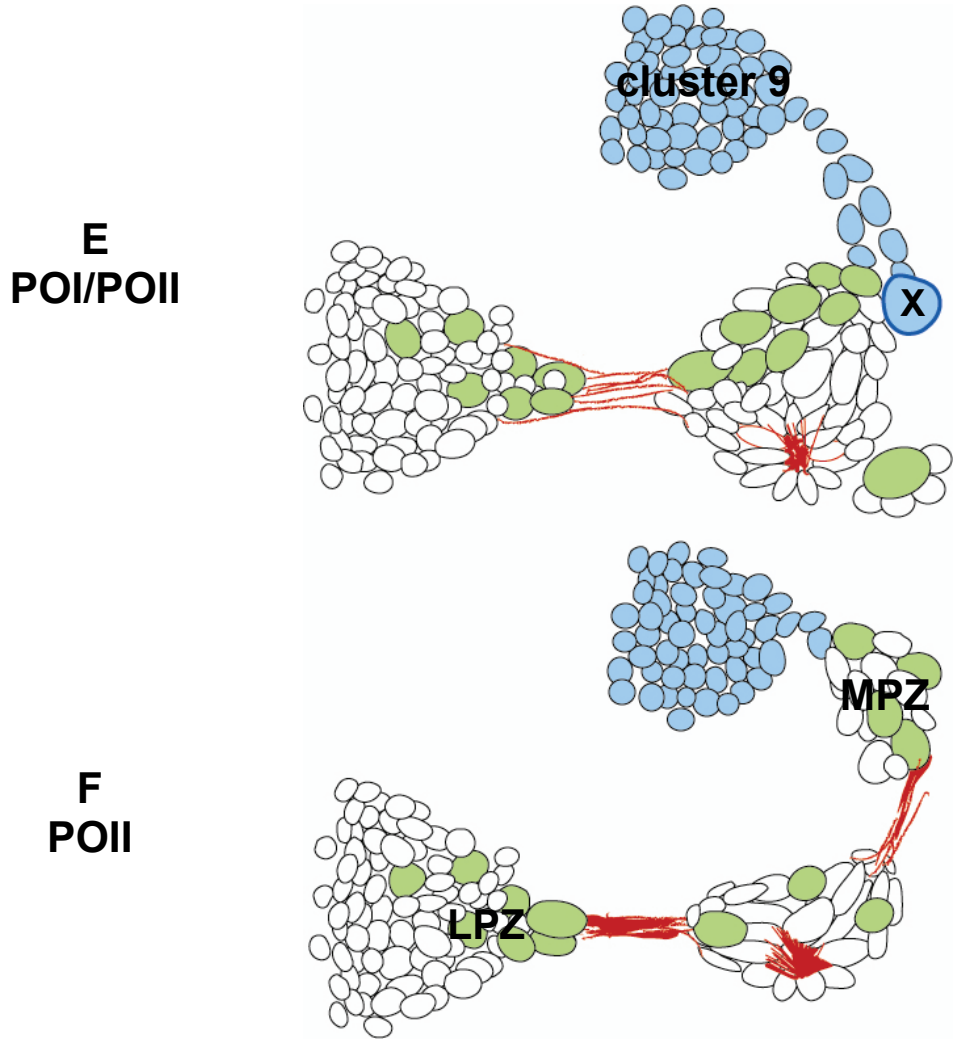
LPZ
cl 10
niche-core


E
POI/POII
cluster 9
X
F
POII
MPZ
LPZ

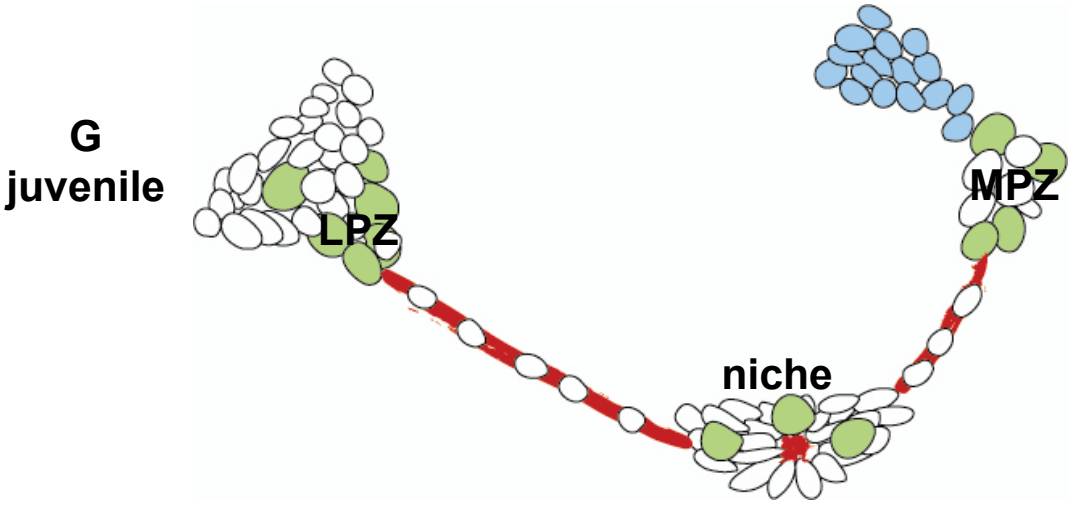

G
juvenile
LPZ
MPZ
niche

Fig. 1- A model of the development of the niche. A: Overview of the embryonic brain just before hatching, when some neuroblasts (NBs) (green circle) and their progeny (white circle) are still present and patchily dispersed in the brain. B: The deutocerebrum region (red square in A) shows a dispersed group of cells, which, during hatching, proliferate and take shape by stretching towards the midline. C: POI stage. The **proto-niche** starts to form. Filaments rich in tubulin (in red) give rise to a central channel-like structure, called "**pore**". The **neuroblast X** (X) is also present and gives off progeny to cluster 9. During POI stage (lasting a couple of days), the proto-niche takes shape, the structure becomes more definite and two main parts are discernible: the **cap** and the **niche-core**. The proto-niche, together with the stream, LPZ and cluster 10, forms the **Deutocerebral Proliferative System**. E: between POI and POII stage, cap cells together with neuroblast X contribute to cluster 9. In some specimens, the cap starts to detach from the niche-core. F: POII, neuroblast X is not visible anymore, most of NBs have disappeared from the rest of the brain. The cap has detached from the niche and has been displaced towards cluster 9. From now on it is called MPZ. G: in earlier juveniles, the **niche** appears shrunken in size, elongated in shape, with two or three dividing cells inside, and connected by two streams towards two proliferative zones, LPZ and MPZ.

The niche develops by rearrangement and by proliferation of cells which probably are already in place in the deutocerebrum of the brain. These cells, together with the extending filaments, give origin to the primitive niche (proto-niche) by cellular movements (Fig. 1C). The initial bunch of filaments emerges close to the giant neuron that is considered here the landmark for identifying the position of the niche. Filaments converge to a single point to form a kind of channel-like structure while the surrounding area is still without any visible boundaries. However, the lateral proliferation zone (LPZ), associated with cluster 10 and the proto-niche, is never separated but it is joined by the migratory stream that gets thinner and thinner as development proceeds. During development of the niche, this stream is always filled with cells that simply represent the umbilical cordon which keeps the lateral proliferation zone associated with cluster 10 and the forming niche joint together. At the very beginning of the POI stage, the proto-niche is in its infancy but two main parts of its structure start to differentiate: the central bunch of cells surrounding the filamentous channel-like structure (the **niche-core**) and the group of mitotically active cells placed on top of it (the "**cap**") (Fig. 1C). Mitotic activity is in fact visible in the cap of the niche indicating a proliferative activity. During POI which may last up to 2-3 days, the proto-niche turns into a more defined structure in which the hub of cells (the "niche-core") wrapped by filaments that form the central cavity and pore, and a multi-layer of proliferative cells (the "cap") on top of this core become clearly distinguishable (Fig. 1D). The proto-niche, together with the LPZ-stream, LPZ and cluster 10 forms the **Deutocerebral Proliferative System** (Fig. 1D). Within the cap, cells proliferate randomly causing a dispersion of daughter cells. In fact, it is not possible to

trace back the history of single cells and their position. As the brain expands in size, the deutocerebral proliferative system stretches out transversely. The migratory stream lengthens as the LPZ and the protoniche move apart. The migratory stream represents a band of cells, wrapped by tubulin-rich filaments, that shrinks in diameter as development proceeds. By the end of juvenile stage, it will look like a collar with single cells lined up like pearls. On the other medial side of the niche, cluster 9 has been shown to be present and fed by new-born cells, but the medial proliferation zone (MPZ) as seen in adults (Sullivan et al., 2007a) is not present yet. Two systems have been shown to contribute progeny to cluster 9: the cap of the proto-niche and one single neuroblast located between the proto-niche and the giant neuron. In fact, two streams of newborns cells end up in cluster 9, one originating from the cap and one coming from the single neuroblast (neuroblast X). Concerning the term "neuroblast" (NB), as well as the concepts of stem cells, progenitors and precursors (treated later in the discussion), some considerations are necessary. The size and shape of a cell are usually considered not sufficient and adequate requirements for defining a NB as such. Nevertheless, we noted that this specific progenitor does not only show characteristic morphological features (round and bigger size compare to other cells, and progeny of smaller cells associated) but its presence and activity is also part of a stage (POI) in which many other neuroblasts are patchily dispersed and active elsewhere in the brain. For these reasons, that cell has been named "neuroblast X". Cleavage plane and asymmetric division are usually good indicators for progenitor cells and future studies could address additional questions about the identity of this cell. After the POI stage, all randomly dispersed NBs disappeared from the brain together with the neuroblast X. Concerning the cap of the proto-niche, from results of the pulse chase experiments, it has been deduced that newborn cells from the cap contribute to both cluster 9 and the niche-core. In fact, newborn cells appear not only in the stream towards cluster 9 but also within the niche-core. The niche-core contributes locally to its own construction as well.

During the transition from POI to POII stage, the cap begins to detach from the niche-core, moving towards cluster 9 to eventually form the second active proliferative zone (MPZ) (Fig. 1E). During these stages (end of POI, beginning of POII), it can be suggested that, upon specific molecular signals, some of the proliferative cells which

were part of the proto-niche perhaps by establishing contact with the surrounding niche-cells by cadherin, become stem cells (see below about definition of stem cells). We have no data on this issue but during later phases of the niche development, adherent junctions by cadherin have been shown to appear around the central "bull's eyes" (the central channel-like structure, the pore) (Sullivan et al., 2007a). My suggestion is that cadherin produced by the niche-core establishes a contact with primordial stem cells, by orienting stem cell division and holding the stem cells close to the maintenance signals of the niche. Alternatives to this view will be discussed below.

In POII, the cap has achieved a new position and a new identity. It is now completely detached from the niche-core, it has moved away from it and from now on it is called Medial Proliferative Zone (MPZ) (Fig. 1F). The proto-niche without the cap is called "niche". Some NBs are still present in the brain, NB-X on the contrary is not visible anymore. Whether the NB-X has ceased its function and disappeared or has become part of the MPZ is not known.

The juveniles phase of neurogenesis in the crayfish *Procambarus clarkii*, together with adult phase, has been monitored by Song and colleagues (2009) and compared with results from Sullivan and colleagues (2007a, b) (Fig. 1G). It has been convincingly shown that during adulthood of *Procambarus clarkii* newborn cells migrate along the migratory streams, from the niche towards LPZ and MPZ (Sullivan et al. 2007a, b). In the current project, how the niche works during the juveniles phase has been only partially investigated, because of the already existing literature about it. However, results of the present study help in filling some gaps of the previous studies. My data are, to some extent, in accordance with Song and colleagues (2009) regarding the continuous proliferation of LPZ during all early post-embryonic stages (POI, POII and early juveniles). The LPZ shows a continuous and constant local proliferation, apparently not dependent by the ongoing formation of the proto-niche. The MPZ in the present study was shown to form only after the formation of the niche when the LPZ is already in place. This explains and clarifies why data from juvenile's migration of cells (Song et al., 2009) and adult migration of cells (Sullivan et al., 2007a) do not match. In juveniles, contrary to adults, the MPZ has just formed by separation of the cap from the niche-core and in some specimens of the Marbled crayfish it appeared just slightly detached from the

niche-core but not completely separated and thus not entirely in place. Data of the present study plainly demonstrate that, while the migratory stream between the proto-niche and LPZ is present since the beginning of the whole process and gets thinner and defined while the brain enlarges, the stream between the proto-niche and MPZ emerges only after the detachment of the cap from the niche-core (POII-Juveniles). Until that moment, it is not possible to talk about a "migratory stream towards MPZ" or MPZ, because neither the stream nor MPZ are present. On the other hand, in adults, the whole deutocerebral proliferative system (LPZ, niche, MPZ and the streams) is formed, defined and functioning (Sullivan et al. 2007 a, b, Zhang et al. 2009, Song et al. 2009). Regulation of the system has still to be fully understood, though.

5.3.2. A question of identity: who is who?

Definition of "niche" is not an easy task. One of the requirements for a structure to be called "niche" is its persistency even in the absence of stem cells. Jones and Wagers (2008) support the hypothesis that niches exist whether or not stem cells are present to occupy them. Theoretically, exogenous cells could even be added directly within the niche and function as stem cells (Spradling et al., 2001). In the *Drosophila melanogaster* ovary and testis, the somatic component of the gonad, together with cells that will eventually be added to the niche, takes shape in the absence of GSCs (Margolis and Spradling, 1995). In mammalian testis, Sertoli cells support spermatogenesis, including SSC (spermatogonial stem cells) self-renewal and they are present and maintained even in aspermic testes (Li and Xie, 2005). Not only, in the haematopoietic system, multiple niches are supposed to exist and they are thought to provide distinct regulatory functions for self-renewal and differentiation. These hypotheses are based on earlier studies by Wolf and Trentin (1968) that showed how microenvironments (niches) can determine cell differentiation outcomes in the haematopoietic system.

Aware of these facts, it could be proposed that not one, but three diverse niches are present in each deutocerebrum proliferative system of the crayfish brain: two active niches (so called LPZ and MPZ) in which stem cells give continuously rise to neurons, and one quiescent niche which appears later and acts as storage-replacement-niche of stem cells, to replace those in the active niches that age. The third niche is necessary for

replacing stem cells in the other two niches because they get old and so it works as support, as storage of stem cells. Daughters migrate (regularly or not? This is also a crucial point that should be accurately evaluated) along the pathways to the other niches before proliferating and contributing to the cluster 9 and 10. Migrating daughters may compete with the resident stem cells in LPZ and MPZ for niche occupancy or they simply replace some old-dead stem cell. The "storage niche" may also act as a signaling source that maintains the undifferentiated state of "primordial" stem cells (PSCs) inside the niche, by maintaining their capacity to populate the active niches as stem cells.

Why talking about three different niches? Because the LPZ exists since time before the appearance of the niche and keeps working also after embryogenesis, at least until juveniles-adult stage without any contribution from the niche. Its local proliferation of cells is constant during early post-embryonic development, and even in adulthood is always present. The LPZ also shows a structure defined by the tubulin or glutamine synthetase staining. Moreover, it does not disappear as we could expect from an embryonic structure but lasts life-long. So it is a defined group of cells, existing before the niche and apparently able to work independently of it, at least until the niche contributes to adult neurogenesis as well. What emerges from my data is that the niche does not suddenly appear to form the LPZ and MPZ. On the contrary, clusters 9 and 10 are fed by progenitor cells before the appearance of the niche that shows up and takes shape only at the end of POII stage and starts working when the animal reaches the juveniles-adult stage (Song et al., 2009). So, our current model of development of the deutocerebral proliferative system considers the LPZ and MPZ not simply as proliferative zones depending on the niche but as defined mitotically active niches. The third niche derives from cells already present in the deutocerebrum of the brain and forms while cluster 10 and 9 are already in place and fed by independent mechanisms (NBs or other progenitor cells). The niche starts working later on during development and probably to provide stem cells in the other two niches which are gone lost or are dead. This is perfectly in range with the description of a typical niche (stem cells hosted in a microenvironment). As summarized by Jones and Wagers (2008), there are distinct functional classes of niche, "each specialized to sustain the unique functions of particular tissues". This means that, in spite of some general principles and roles that can be derived

by diverse studies, each stem cell niches may represent a special case in that specific tissue of that specific species of animal. As it will be explained below, niches may show high flexibility and different dynamics according to the needs of the tissue in which they reside. In the Marbled crayfish for instance, we witness the presence of a network of three niches that, one after the others, takes shape from the same first cluster of cells and apparently they are able to work independently for sometime and by different dynamics, although connected to the whole system. For simplicity and coherence to the rest of the text, the known published nomenclature will be used (LPZ, niche, MPZ).

Moreover, the "three-niches" model could give an explanation for the replacement of stem cells. So far, as hypothesized by Sullivan and colleagues (2007b), replacement of stem/precursor cells may occur via the central cavity which has been proven, by injection of dextran, to be in contact with the vascular system. More data are surely needed to clarify how replenishment of stem cells takes place.

5.3.3. Identity of stem cells

Not only niches, defining stem cells is a tricky task as well. Before going into details on the hypothesis underlining the identity of cells responsible for adult neurogenesis in the Marbled crayfish, some thoughts on types of dividing cells are warranted. Three types of cells with proliferative characteristics can be distinguished: progenitors, precursors and stem cells. As mentioned in the introduction, debate on the real meaning of these types of cells is still open and specific investigations are necessary to clearly define the specific features of each of them and in which way they are related to each other. Among the broad literature on stem cells from different species and tissues, in order to define these cells and apply the concepts to my studies, I refer to one of the most recent definitions given by Martinez-Agosto and colleagues (2007) regarding the hematopoietic tissue, the best described stem cell population in mammals (Fig. 2). It is important to define the source from which the definition is taken because a standard nomenclature is not available yet and misunderstandings are likely to occur.

Progenitors are embryonic mitotically dividing cells that can be both pluripotent (i.e. blastomeres in mammalian embryos. In other sources, these cells are called "pluripotent embryonic stem cells" able to give rise to all types of cells) and multipotent (ectoderm or

mesoderm) (Fig. 2a A-C). At later developmental stages, progenitor cells exit the mitotic cycle. From progenitor cells, precursors and stem cells arise (Fig. 2a D, E). Precursor cells can be still multipotent but after a while they differentiate (Fig. 2a D). Stem cells develop from progenitor cells that are "prevented from exiting the mitotic cycle by microenvironments, called niches. In the adult organism, stem cells undergo asymmetric divisions and produce mitotically active daughter cells also called progenitors" (Martinez-Agosto et al., 2007) (Fig. 2a F). Thus, stem cells are produced in embryos and persist in specific niches where they can remain mitotically quiescent for long period of time. Actually, they can show a different dynamics of activity: to expand their number they self-renew by symmetric division, to maintain their number they produce rapidly dividing progenitors by asymmetric division (Martinez-Agosto et al., 2007) (Fig. 2b)

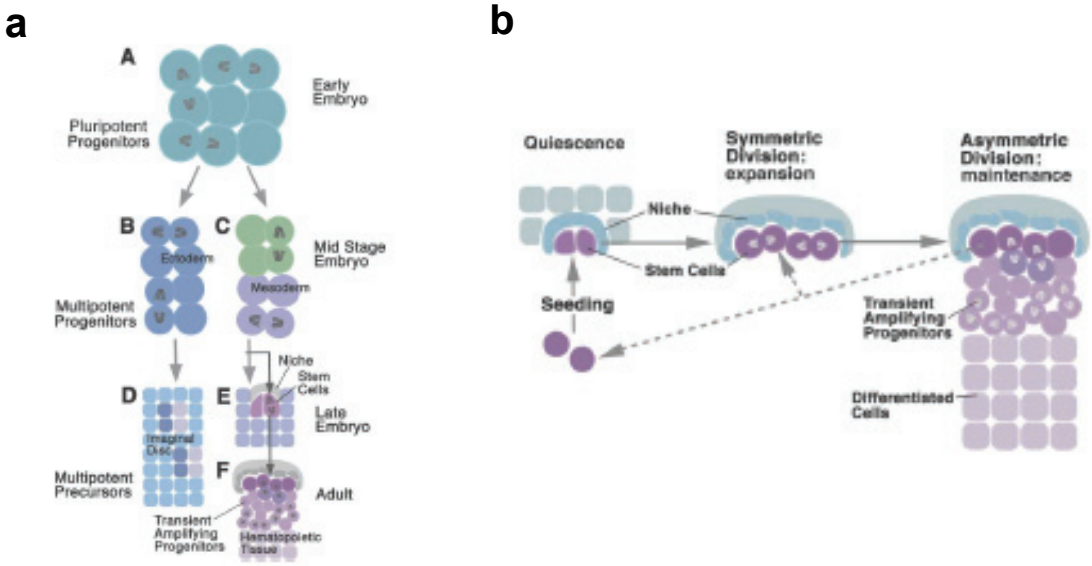


Fig. 2- a) Stem cells in the context of development. A–C: embryos consist of mitotically dividing cells called progenitors. Progenitors can be pluripotent or multipotent. D: at later developmental stages, cells exit the mitotic cycle. Generally called precursors, these cells can still be multipotent (e.g., cells of imaginal discs in *Drosophila*). At some point precursors become committed to a particular fate and differentiate. E: stem cells (e.g., HSCs, hemapoietic stem cells) develop from embryonic progenitors that are prevented from exiting the mitotic cycle by specific microenvironments, called niches. F: in the adult organism, stem cells undergo asymmetric cell divisions and produce mitotically active daughter cells also called progenitors ("transient amplifying cells") (modified by Martinez-Agosto et al, 2007).
b) Stem cell dynamics. Stem cells are generated in the embryo. They persist in specific niches where they can remain mitotically quiescent for long periods of time. Stem cells expand their numbers by symmetric division. They can also maintain their numbers and produce rapidly dividing progenitors by asymmetric division (from Martinez-Agosto et al., 2007).

Going back to arthropods, in *Drosophila* neuroblasts give rise to all neural cell types and are present only momentarily during development as they disappear during metamorphosis (Urbach and Technau, 2004). By contrast, in cricket, NBs persist throughout the life span of the insect (Cayre et al., 2007). So, are NBs stem cells, progenitors or precursors? Apparently, persistence through life is not a must for being a stem cell. Spradling and colleagues (2001) underlined, "the true nature of stem cells can be learned only by discovering how they are regulated". This concept is at the base of the "niche hypothesis" proposed by Schofield (1978): location, rather than a specific pattern of genetic expression, characterizes stem cells. That is why the structure of the niche is considered essential for the stem cells and they can not be treated separately. To resume, according to Martinez-Agosto and colleagues (2007) a stem cell must have:

- multipotency and asymmetric cell division as means to give rise to multiple cell types
- quiescence and slow self-renewal as means to allow long life span
- niche dependence

These general criteria may define a stem cell but, as mentioned before, stem cells are generated during embryogenesis and are present in adults, thus there are two types of stem cells: embryonic and adult, each of them with other peculiar characteristics. For instance, adult stem cells can show different "forms" which reside in niches of specific organs (brain, ovary, testis) (Li and Xie, 2005). Regulation by extrinsic factors and intrinsic genetic programs characterizes each stem cell of a distinctive niche. One lesson comes again from the haematopoietic system in which embryonic and adult stem cells show different properties, due to differences in their cell surface marker expression and activity (Bowie et al. 2006, 2007). These principles make the identity of stem cells not an easy issue. They are able to both types of division (symmetrical and asymmetrical), they can show both slow and fast mitotic activity and they are closely associated to progenitor cells from which they derive and that, at the same time, represent their progeny (Fig. 2).

Keeping in mind these premises and talking about the Marbled crayfish, during the development of the niche different scenarios may be feasible and they may involve either embryonic or adult diverse mitotically dividing cells types. Surely more and diverse experiments are required to clarify the identity of each cell of each compartment (cap,

niche-core, LPZ, MPZ, Neuroblast X) but some hypothesis can be proposed. First of all, because a niche with proliferative cells inside has been proved to appear and exist on the ventral surface of the brain of crayfish, in the present study stem cells refer to such cells, those within the adult niche.

1) Stem cells within the niche derive from some local multipotent progenitor cells. As seen in Chapter 2 of embryogenesis, in the brain of crustaceans ectoteloblasts are not present but NBs are in place since the germ disc appearance. If we establish that NBs are the first cells in the early embryonic brain, then NBs are the multipotent progenitors. NBs of the deutocerebral proliferative system give rise to the development of the niche and are eventually present in the cap of the proto-niche. So, one hypothesis could be that some of these embryonic NBs move inside the niche-core and, by effect of the mature niche that prevents the progenitor to further divide, it becomes a stem cell. This version implies that, before niche formation, cells of the deutocerebral proliferative system (primordial stem cells, PSCs) proliferate as progenitors and are undifferentiated. As the niche starts to form, PSCs divide into two distinct subpopulations: precursors which will keep on dividing by multipotency and eventually differentiating to form the niche structure (i.e. the cap) and PSCs that acquire stem cell identity.

What happens next? The current project did not deal with adult specimens, only with first post-embryonic stages to which the model of Sullivan and colleagues (2007a) cannot be applied. However, some thoughts are here presented, as ideas for future experiments. According to Sullivan and colleagues (2007a), the adult niche buds off cells that migrate towards LPZ and MPZ where they divide two or three times to generate immature neurons which eventually differentiate into olfactory interneurons. Moreover, Zhang and colleagues (2009) observed symmetrical divisions within the niche. My hypothesis is that stem cells in the adult niche act as storage niche and divide symmetrically only to expand their number when some of them migrate towards LPZ and MPZ that, as stated before, could be considered “niche” as well. These migrating stem cells are budded off to replace stem cells in the PZs that are characterized by asymmetrical divisions (Pl. 5.20). Here they divide by producing each time progenitor cells which in turn produce precursor cells. Because mitotic activity has been seen also in the region surrounding the LPZ (Pl. 5.19), it can be suggested that precursor cells are displaced toward cluster 10, through the

Transit Zone, where they differentiate into neurons while progenitor cells, together with stem cells, keep on dividing in LPZ. This model is mostly in accordance with Sullivan and colleagues (2007) but more emphasis on the identity of cells is given and the niche in between the two PZs is suggested to be a storage niche, rather than a dynamic niche.

2) Stem cells do not derive by local progenitors but they were committed to their fate *a priori* somewhere in some tissue and entered the niche *via* vascular system (the central cavity has been shown to be connected to the vascular system. See Sullivan et al., 2007a). Another alternative is that there are stem cells in other regions of the brain that somehow get into the niche.

In consequence of these hypotheses, the question "where do stem cells come from?" arises spontaneously. In *Drosophila* the primordial germ cells which form the stem cells of the ovary come from a distinctive lineage of cells set aside from the soma early in the development (Gonzalez-Reyes, 2003). They start their lives as pole cells and they are committed to germ cell fate at the time of their formation due to some maternal inheritance made of specific proteins, sufficient for germline determination. These cells migrate to the gonads where they will become stem cells after connection by cadherin to the cap of the niche. If the stem cell loses the contact, it becomes a daughter cell (precursor cell) which leaves the niche and thus has to be replaced. Unfortunately such question about provenience of stem cells in the Marbled crayfish brain cannot be answered by our data.

In order to avoid further misunderstandings due to the same nomenclature, cap cells of *Drosophila* are part of the mature adult niche while in crayfish cap cells are present only in the developing niche, not in the adult form. In this specific context, cap cells of *Drosophila* refer to the crayfish niche-core cells.

5.3.4. Are niche-stem cells glia cells?

The hypothesis brought up by Sullivan and colleagues (2007a) (glia identity of niche cells) and confirmed by Zhang and colleagues (2009) is mainly based on both anatomical features of cells identified by gluthamine synthetase (GS), a marker for glia cell, and analysis of the cleavage plane. By using an intracellular dye, these authors showed that cells within the niche (the niche-core) have a spindle-like shape and cytoplasmatic

extensions. Because the niche is GS positive, Sullivan and colleagues (2007 a, b) concluded that these elongated niche cells are progenitors (authors do not use the term "stem cells"). Zhang and colleagues (2009) confirmed this finding by combining proliferation analysis with experiments on cell division of neuronal precursors and nucleokinetic analyses and thus supported the glia hypothesis *versus* the neuroblast hypothesis (large, spherical cells inside the niche) sustained by Song and colleagues 2009. Nucleokinesis is the process involving cytoskeleton reorganization that brings morphological changes of the nucleus and allows migration of cells during development. By such studies, Zhang and colleagues (2009) gained insight into the activity of progenitor cells residing in the niche and suggested a model of migration of daughter cells. Authors provided an explanation of the spindle-shaped nucleus *versus* the large round nucleus, claimed by Song and colleagues (2009) as proof of NB identity. They suggested that elongated cells, in order to divide, reabsorb the cytoplasm that was previously extended from one side to the vascular cavity and to the other side towards the proliferation zones: by loosing the cellular extensions, nuclei become spherical. So, putative NBs observed by Song and colleagues (2009) may actually be cells that have loosened their elongations and are about to enter cytokinesis. Moreover, they showed that all divisions are symmetrical and that the cytoplasm of daughter cells labels for gluthamine synthetase.

In the present study, the use of gluthamine synthetase (GS) immunohistochemistry to identify the niche and eventually the stems/progenitor cell was not decisive. The antibody turned out not to be highly specific for the niche and precursor cells, at least compared to the clearer labeling by tyrosinated tubulin. In any case, it can be argued that, by simply using GS, it is not possible to provide unambiguous evidence of glia cells as main components of the niche. It is also useful to recall that usually (as seen in the insect literature) the roundness and large size coinciding with a lack of cellular extensions are common features of stem cells (i.e. neuroblasts). Moreover, few (two or three) big stem cells have been usually seen residing within niches of other species, not more (i.e. *Drosophila*). Aware of these arguments, the hypothesis proposed by Sullivan and colleagues (2007a, b) and Song and colleagues (2009) that, in *Procambarus clarkii*, dividing cells are numerous, densely packed within the niche, elongated and with cellular

expansions may appear questionable. More data are surely necessary to clarify this matter especially in terms of nomenclature and definition of progenitor/precursor/stem cell. Our data only partially contributed to the current dispute between the two models (Sullivan et al. 2007 and Zhang et al. 2009). The present study in fact focused on the development of the niche and the appearance of the first sign of activity. Nevertheless, current results together with the most recent literature suggested the following scenario that could be applied also to *Procambarus clarkii*: elongated GS-positive cells within the niche are not stem cells. They get in contact, by cytoplasmatic filaments, with two-three stem cells to anchor them. Dansereau and Lasko (2008) in fact reported that, in the ovary niche of *Drosophila*, cells forming the cap of the niche are characterized by cytoplasmatic processes which are used to isolate and get in contact with stem cells. In crayfish, stem cells may be represented by the two "putative" NBs, claimed by Song and colleagues (2009), which are located in each half of the niche of *Procambarus clarkii*. The identity of those two stem cells remains unknown, Song and colleagues (2009) define them as NBs but, as discussed above, they could actually derive from some local NBs or coming from external sources (blood).

5.3.5. When does/do the niche/niches replace NBs?

In other words: when does the post-embryonic proliferative system start working? Recent studies in *Drosophila* clearly showed that one of the requirements for stem cells, to work as such, is the tight association of stem cells to the "cap" cells (as said before, cap cells in *Drosophila* are here considered equivalent to the niche-core cells of the adult niche) (Xie and Spradling 2000, Song et al. 2002, Song et al. 2007, Morrison and Spradling 2008). For these reasons, it could be hypothesized *a priori* that during the formation of the niche, stem cells are not active (and so the niche itself does not work) because either they are not yet inside the niche or the association by cadherin between progenitor cells and the niche-core cells has not been established yet. According to the current models on stem cell niches, stem cells must be anchored to support-cells (according to the species they have been named differently: cap-cells, hub cells, distal tip cell, etc). In our case, support cells are the niche-core cells. Studies on stem cells from different animals and diverse organs (*Drosophila* ovary/testis; *Caenorhabditis elegans* distal tip, mammalian

bone marrow, skin/hair follicle; intestine, brain and testis) showed that extracellular signals and intrinsic genetic programs within the stem cells control the stem cell function (Li and Xie, 2005). Moreover, stem cells and the support cells may co-develop, "with the emergence of each being dependent on proper specification, localization and interactions with the other" (Jones and Wagers, 2008).

Stem cells niches have been recorded to be dynamic and flexible as regards to function regulation. For instance, in the intestine and epidermis, stem cells function continuously to replace mature cells with a restricted lifespan (Jones and Wagers, 2008). On the contrary, in the skeletal muscle, the progeny of stem cells (muscle fibers) are long lived and only when needed (after damages) the niche provides replacement (Schultz et al., 1978). In some systems, like the blood, both active and quiescent regulation is necessary because the haematopoietic stem cells (HSCs) have to maintain both a daily blood cell production and the capacity to respond to injury (Morrison et al., 1997). Additionally, studies on *Drosophila* ovary demonstrated that niches depleted of stem cells maintain the ability to support incoming cells, and are able to promote self-renewal and proliferation of cells that usually do not reside inside a niche (Xie and Spradling, 2000).

To sum up, a niche may show high plasticity in regulation and many aspects have been discovered in tissues of other species. In the present study, the appearance of the niche and its complete development has been shown. Migration of newborn cells has been recorded by pulse-chase experiment that showed how daughter cells, during POI and POII stages, moved from the niche "area" towards cluster 9. As demonstrated, the cap of the niche (what will become MPZ) is responsible for such production of new cells, together with a neuroblast that disappear after few days after the hatching. The LPZ (and thus cluster 10) appeared always independent by the ongoing formation of the niche. These results showed that the proto-niche does not work. In juveniles, no pulse chase experiments was performed but few (two or three) BrdU positive cells appeared within the niche. In adults, it has been shown (by pulse-chase experiments) that the niche works by sending off cells that migrate along the streams towards LPZ and MPZ (Sullivan et al. 2007a, b).

How the brain niche/niches are regulated in the brain of crustacean has still to be understood as well as the provenience of stem cells, their identity and their activity.

Results presented in this thesis represent a start for future investigations on this research topic that is extremely challenging and intriguing.

5.3.6. Methods to study adult neurogenesis

One issue to be mentioned concerns methods by which neurogenesis can be studied. In the past and nowadays, the use of bromodeoxyuridine (BrdU) has been extensively applied for detecting "proliferation" of cells (Nowakowski et al., 1989). In spite of its prominent use, the use of BrdU represents one of the main limitations in determining and understanding the role of newborn neuronal cells of the adult brain as well. In fact, BrdU is a thymidine analog that incorporates DNA of dividing cells which means that BrdU is not a marker for cell proliferation and neurogenesis. It is a marker for DNA synthesis. Furthermore, BrdU is a toxic and mutagenic substance (Morris, 1991) and these features may deceive the study of adult neurogenesis (Taupin, 2007). Labeling with BrdU is, indeed, necessary but not sufficient to prove that a cell has divided. DNA repair and turnover, for instance, are part of nuclear activities present also in "old" neurons that do not proliferate but that would result BrdU positive (Rakic, 2002). Another issue is represented by the use of specific neuronal antibodies to prove that newborn cells are neurons. It has been shown that many neuron-specific markers label other classes of cells as well (Deloulme et al. 1996, Sensenbrenner et al. 1997).

Accuracy in determining the exact life stage of the examined specie is another important matter. For instance, in Song et al. (2009), the POII stage of *Procambarus clarkii* has been misidentified with POI (animals still connected to the mother by anal thread; Sandeman and Sandeman, 1991). This type of mistake should not occur in order to not misinterpret results on similar/same species.

5.3.7. Future directions

Development of the niche was followed by relying on immunohystochemical techniques that allowed the identification of the proto-structure and main components of the raising niche. Identifying origin of stem-progenitor-precursor cells and mechanism of recruitment among proliferative cells forming the niche turned out to be more complex and difficult to investigate than it was thought when designing the experiment protocol.

More sophisticated tests will be needed in future to clarify the missing points of our model. What should be done? As suggested by the current study and by stem cells literature, morphological analysis and use of BrdU are not enough for clearly distinguishing the identities of progenitor/stem cells. One method is the cell culture assay which allows the study of stem cells *in vitro* but not *in vivo*. Molecular characterization and fate mapping are considered a must because it is likely that cap-cells and stem cells express different markers and through lineage analysis, by fate-mapping the progeny of stem cells, it would be possible to distinguish them, as it has been seen in other niches (*Drosophila* for instance). The technique is based on the identification of stem cells by molecular marker and afterwards the lineage is followed (Spradling et al., 2001). Unfortunately, specific stem cell markers are not available for many tissues and studies *in vivo* still represent an obstacle to researchers. Discovery of unique cellular and molecular components of stem cells and their niches will provide important insight into the nature of these cells. Analysis of cellular division can also result useful in the study of stem cells. Stem cells exhibit asymmetric division to self-renew (*Drosophila*) but it is unknown whether spindle orientation is essential for other system or not (Li and Xie, 2005). For instance, in the crayfish brain, symmetrical divisions of neuronal progenitors cells have been observed in the niche, where the migratory streams emerge (Zhang et al., 2009). Moreover, as stated by Martinez-Agosto and colleagues (2007) regarding the hemapoietic stem cells in mammals, stem cells are able to perform both divisions according to the needs of the niche. Studying development by analyzing the expression of cadherin can be another critical point. In fact, as explained in this chapter, only after the junction between primordial stem cells (PSCs) and niche cells by cadherin, PSCs become active stem cells and so the niche can work. Before that, apparently the niche does not work in that sense. So, it is essential to understand when exactly stem cells get attached to the niche, acquire the fate as stem cells becoming officially stem cells, and where they come from.

5.4 CONCLUSIONS

In the present study, both development of the niche and mechanisms by which newborn cells are generated *in situ* and transferred to different locations have been analyzed in first post-embryonic stages. Results on the development of the niche ended up in a model which describes the construction of the niche from its first appearance as immature and not working structure until it starts working independently. Results on the function of the niche have been compared with the most updated literature and some new hypotheses have been suggested. The hypothetical mechanism of the crayfish niche proposed by Sullivan et al (2007a) was proposed for adult animals in which the niche has already achieved a mature state. During the development of the niche, first sign of activity does not follow that mechanism, as cluster 10 and cluster 9 are fed not by the niche but by other means: LPZ contributes independently to cluster 10, the cap of the niche and neuroblast X contribute to cluster 9. For these reasons, here it is proposed that the whole deutocerebral proliferative system is formed by three "feeding" niches, two of which (LPZ and MPZ) are independent and active and the third acts as storage and replacement pool of stem cells. Additionally, the glia hypothesis based on the use of the gluthamine synthetase antibody could not be confirmed for animals in POI and POII stages because of the unspecificity of the antibody. The niche structure was in fact stained by tyrosinated tubulin antibody. Hypotheses on the identity of stem/progenitor/precursor cells have been mostly based on the current literature available on stem cells and niches of other species and surely specific investigations tailored on the Marbled crayfish must be carried out to confirm these hypothesis.

Chapter 6

Matherials and Methods

6.1 MATERIALS AND METHODS

6.1.1. Animals

Marbled crayfish embryos were taken from a laboratory culture in which single adult individuals (or one adult together with the juvenile progeny) were kept in small aquaria filled with tap water (approximately 5lt) at 20°C water temperature and a 10-h light/14-h dark regime as described by Seitz et al. (2005). Aquaria were provided with pottery shelters, dried leaves and grainy sand. Water was oxygenated and changed twice a week, animals were normally fed three times a week with crustacean food pellets (TetraMin) and vegetables (carrots and peas) and checked in terms of deaths, molts or freshly laid eggs (Fig. 6.1). For the experiments, animals were staged according to the embryonic development staging system established by Seitz et al. (2005) (see Chapter 1).

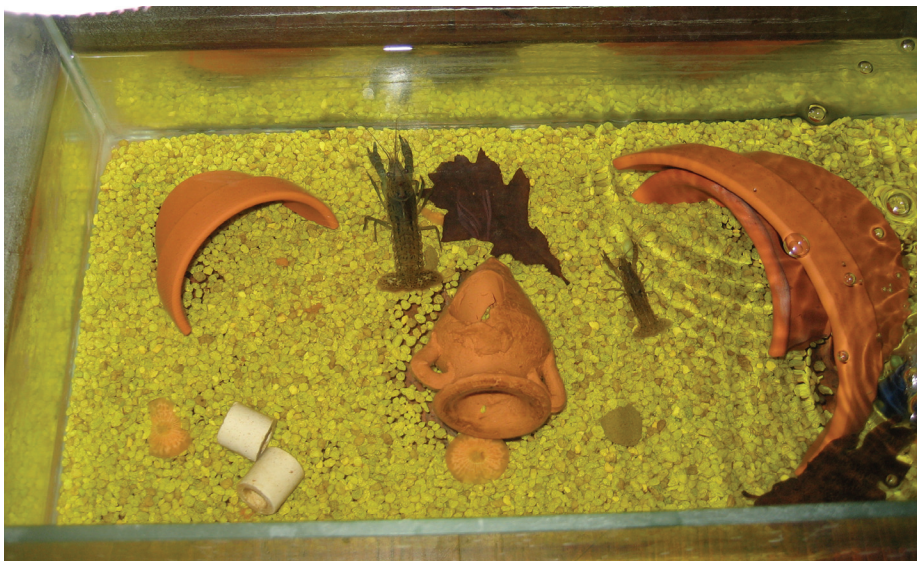


Fig. 6.1- Typical set of an aquarium for the Marbled crayfish. One adult and one juvenile share the same tank in which pottery shelters, food and dried leaves are provided.

6.1.2. Immunohistochemistry

The first publication describing an immunofluorescence technique for detecting cellular antigens in tissues was by Coons and colleagues in 1941 and that can be considered the beginning of immunohistochemistry (IHC). The basic concept behind IHC is that antigens (Ags) (for instance proteins) within tissues can be localized by specific antibodies (Abs) whose primary function is that of specifically binding to antigens in

biological tissues. Afterwards, the primary antibody can be visualized by a colored histochemical reaction (Ramos-Vara, 2005). IHC is widely used in scientific research to understand the distribution and localization of biomarkers and proteins in different parts of a biological tissue, for the diagnosis of abnormal cells such as in tumors and to localize specific molecular markers which are characteristic of cellular events such as proliferation or cell death.

6.1.3. Antibodies

Antibodies (also called Ig, immunoglobulin) are proteins with a structure that resembles the Y, consisting of two identical light chains and two identical heavy chains. There are 5 classes of immunoglobulins: IgG, IgA, IgM, IgD and IgE, sharing the same light chains and characterized by different heavy chains. The most common used in IHC is the IgG (about 75%) (Ramos-Vara, 2005). In general, Abs are made by animals (mouse, rabbit, goat, horse, etc) which are immunized with a specific Ag. The response of the animal is mediated by B cells that bind to different epitopes of the same antigen and produce and secret Abs. Afterwards, Abs recognize and bind to the Ag according to the specific epitope which the Ab was made for. In IHC two types of Abs are used: polyclonal and monoclonal (Ramos-Vara, 2005).

6.1.3.1. Polyclonal antibodies

Polyclonal antibodies are produced in different types of animals (rabbit, horse, chicken and goat) by injecting animals with purified peptide Ag and, after the immune response is stimulated, polyclonal antibodies from different B cells are isolated. Thus, polyclonal antibodies are a heterogeneous mix of antibodies, some even irrelevant, that recognize several epitopes and show higher affinity but lower specificity compares to monoclonal antibodies (Ramos-Vara, 2005). (Fig. 6. 2)

6.1.3.2. Monoclonal antibodies

Monoclonal antibodies are usually produced in mouse. Mice are injected with purified Ag to stimulate the production of antibodies. Cells from the spleen (B cells) are isolated

and fused with cultivated tumor cells from mouse (myeloma cells) because isolated B cells have a limited span life while tumor cells not.

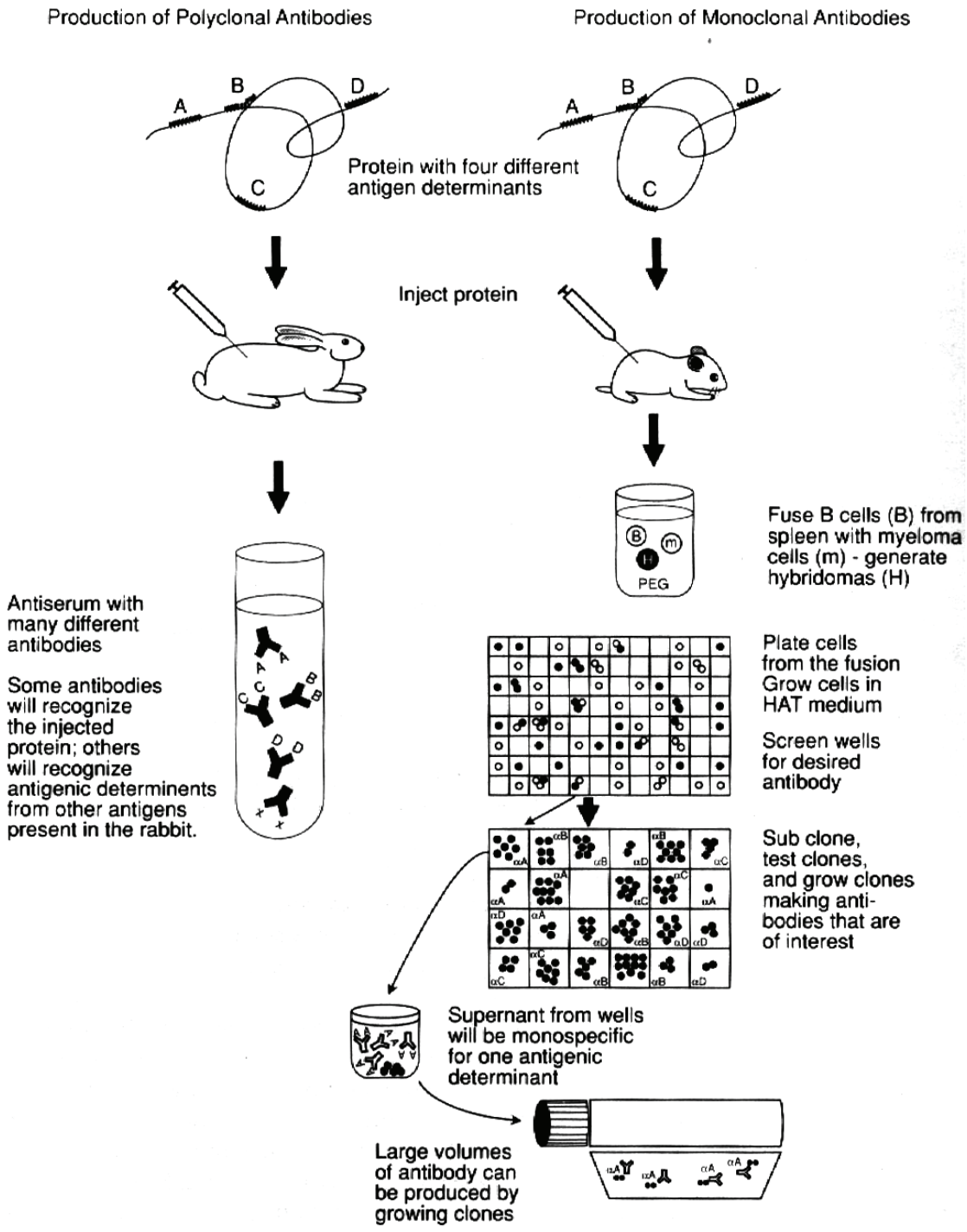


Fig. 6.2- Production of polyclonal and monoclonal antibodies. Explanations are both in the figure and in the text (from Beltz and Burd, 1989).

The hybrid cell produced (hybridoma) is in fact an immortal cells that produces Igs specific for a single epitope and that can be maintained in cell cultures. Hybridomas are thus screened for antibody production. Because of these reasons, monoclonal antibodies are identical and they show great specificity (Beltz and Burd, 1989) (Fig. 6. 2).

6.1.3.3. Primary and secondary antibodies

Antibodies can be distinguished in primary and secondary antibodies. Primary Abs are raised against an antigen of interest and usually are unlabelled. Secondary antibodies are raised recognize immunoglobulins of a particular species of animal and are conjugated to fluorescent agents (Alexa Fluor, for instance), biotin or enzymes. In specific, the secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised (Ramos-Vara, 2005).

6.1.3.4. Detection

Although theoretically simple, the methodology underlining the IHC can be various and visualizing an antibody-antigen interaction can be accomplished in different and complex ways (Fig. 6. 3).

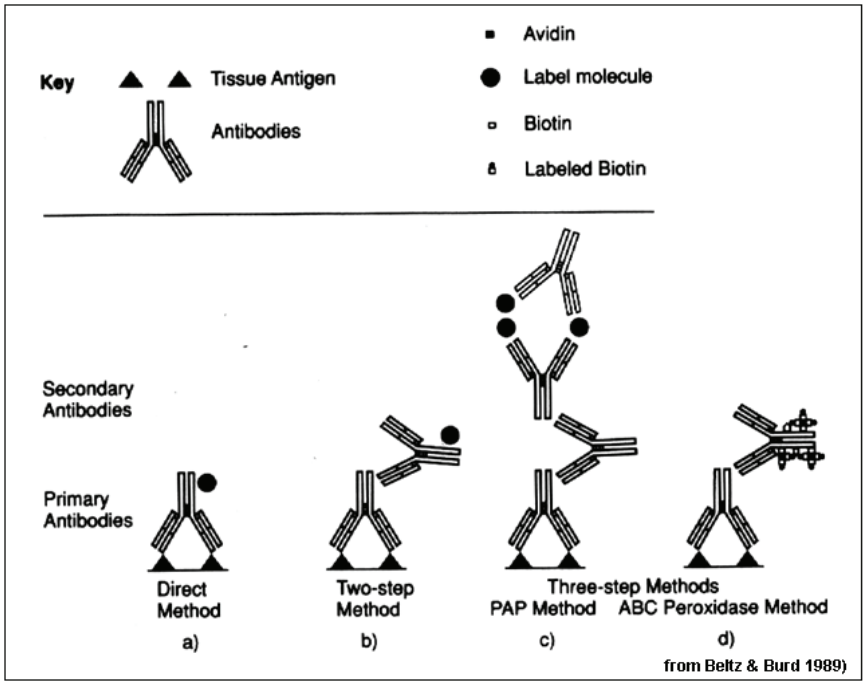


Fig. 6.3- Methods of immunohistochemistry used to detect antigens in biological tissues. More explanations are reported in the text (from Beltz and Burd, 1989).

First applications were based on the so called direct methods that produced quick results but with no much sensitivity (Coons and Kaplan, 1950. In: Ramos-Vara, 2005).
The reaction involves one primary labeled antibody which binds directly to the antigen. The procedure is simple and rapid but it can lack sensitivity due to little signal amplification (Fig. 6. 3a). Because of these limitations, Coons and colleagues (1955. In: Ramos-Vara, 2005) developed a more sensitive method, the two-step method, in which the first layer of Abs reacting with the antigen in the tissue is not labeled while the second layer (secondary antibody), raised against the primary Ab, is labeled (Fig. 6. 3b). The second layer antibody can be labeled with a fluorescent dye such as FITC, rhodamine or Texas red, and this is called indirect immunofluorescence method, or with an enzyme such as peroxidase, alkaline phosphatase or glucose oxidase, and this is called indirect immunoenzyme method. The sensitivity is higher due to signal amplification through many secondary antibody reactions. In fact, three step methods can be also applied in which a third layer is involved. Most commons are the PAP Method (peroxidase anti-peroxidase method) and ABC method (avidin-biotin complex method) (Fig. 6. 3c, d) (Beltz and Burd, 1989).

6.1.4. The confocal microscopy

Biological tissues treated with fluorescent dyes can be seen by using fluorescent or confocal microscopes which, compare to conventional microscopes, provide a more significant imaging improvement. The basic concept of confocal microscopy was conceived by Marvin Minsky during the 1950s (Minsky, 1988). Following his work, David Egger developed the first mechanically scanned confocal laser microscope and published the first images of cells in 1973 (In: Claxton et al., 2005).
Principles and main components of confocal microscopy are represented in Fig. 6. 4. In a conventional fluorescence microscope, the entire specimen is inundated by light from a light source (mercury or xenon lamp). All parts of the specimen are excited and the resulting secondary fluorescence is directly detected by the microscope photodetector and can be viewed in the eyepieces. By contrast, the confocal laser scanning microscope works by illuminating the specimens point by point thus eliminating the out-of-focus light. In more details, a multiple laser excitation sources produce a laser beam that passes

through a light source aperture (pinhole) and then is focused by an objective lens (dichromatic mirror) into a small focal volume on the surface of a specimen (Claxton et al., 2005).The fluorescence emitted from points of the specimens goes back through the dichromatic mirror and is focused at the detector pinhole aperture. The photomultiplier detector transforms the light signal into an electrical one that is recorded by a computer (Fig. 6. 4).

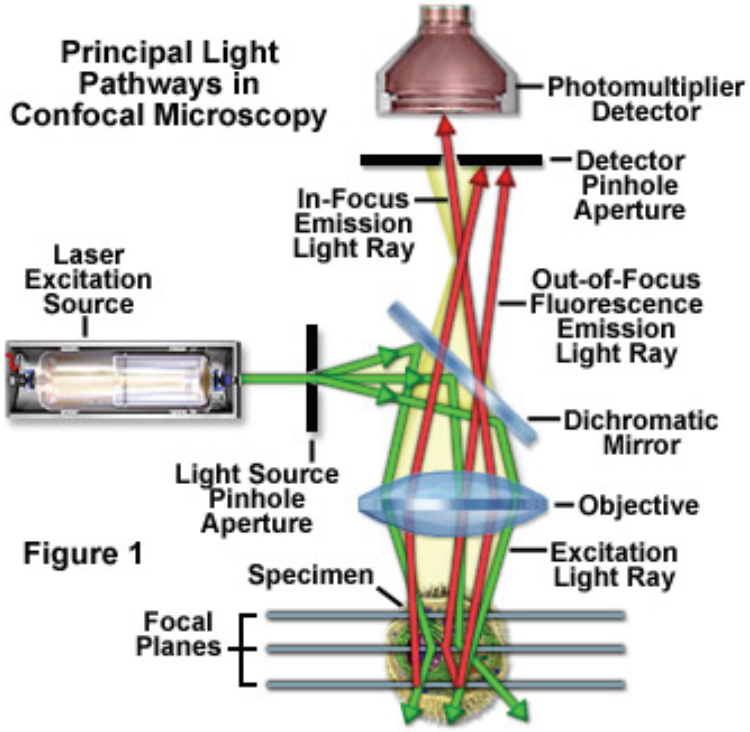


Fig. 6.4- Schematic diagram of the optical pathway and principal components in a laser scanning confocal microscope (from Claxton et al.,2005).

The primary advantage of laser scanning confocal microscopy (LSCM) is the capacity to acquire in-focus images from selected depths, a process known as optical sectioning that produces thin (0.5-1.5 micrometer) optical sections. Images are acquired point-by-point and reconstructed by the computer, allowing three-dimensional reconstructions of structurally complex objects. Contrast and definition are improved over widefield techniques due to the reduction of the background (Claxton et al., 2005).

6.1.5. Applying immunohistochemistry to neurobiology

In the current project, the aforementioned concepts and techniques were applied to investigate the development of the brain during and after embryogenesis in the Marbled crayfish, according to the aims of the whole project.

Commercial antibodies, both polyclonal and monoclonal, have not been generated with intend to be effective in our freshwater crayfish, at least so far. For this reason, before making use of any primary antibody on a specific species not mentioned by the manufacturer of the Ab, a careful investigation and characterization of the desired Ab is necessary. The characterization of antibody specificity in fact requires demonstration that the antibody binds only to that specific protein (Saperd and Sawchenko, 2003). Determination of the Ab specificity can be done by diverse methods, one of these is Western Blotting, used to detect specific proteins in a given sample of tissue. It uses gel electrophoresis to separate proteins by the length of the polypeptide the 3-D structure of the protein. The proteins are then transferred to a membrane (nitrocellulose) where they are detected using antibodies specific to the target protein (Saperd and Sawchenko, 2003).

6.1.6. Primary antibodies characterization

6.1.6.1. Monoclonal Anti-Gluthamine synthetase antibody **(**1:100; BD Biosciences Pharmingen; No. 610517)

As reported in the product-sheet "Gluthamine synthetase is the enzyme that catalyzes the amination of glutamic acid to form glutamine. It is found in mammals as an octamer of 45kDa subunits". Glutamine synthetase activity can be used as marker for astrocytes (from the product sheet). The mouse anti-glutamine synthetase antibody was raised against sheep glutamine synthetase. For Western blots of *P. clarkii* brain homogenate, the antibody stains a single band at ~44 kDa (Sullivan et al., 2007a). This immunoreactive peptide has a molecular weight similar to that of glutamine synthetase in the brain of the spiny lobster *Panulirus argus* (~42 kDa; Linser et al., 1997). Linser and colleagues (1997) confirmed that the spiny lobster brain produce gluthamine synthetase. In fact, immunohistochemical staining of sectioned lobster olfactory lobes and sensory sensilla showed strong reactivity in specific cells (Linser et al., 2007). Comparison of the GS immunostaining pattern with that for FMRFamide, a marker of neurons in invertebrate neural tissues, showed that GS is glial-specific in lobster neural tissues as it is in vertebrates (Linser et al., 1997).

6.1.6.2. Monoclonal anti-Tyrosinated Tubulin (1:1000; Sigma; T9028)

Microtubules are mainly formed by tubulin which is a heterodimer consisting of α-tubulin and ß-tubulin, subject to specific post post-translational modifications at the C termini of both units (Erck et al., 2005). One of these modifications, the tyrosination cycle, involves the cyclic removal of the C-terminal tyrosine of α-tubulin by a carboxypeptidase and the readdition of a tyrosine residue by the tubulin-tyrosine-ligase (TTL). The TTL activity is highest in the brain of mammals and its suppression causes disorders in neuronal organization (Erck et al., 2005). The tyrosination cycle is conserved among eukaryotes and produces two tubulin types: tyrosinated α-tubulin (Tyr-tubulin) and detyrosinated α-tubulin (Glu-tubulin), which lacks the C-terminal tyrosine. In fact, the genes encoding α-tubulin ends in a tyrosine codon indicating that the intact gene product is tyrosinated (Tyr-Tu). In cultured cells, Glu-tubulin is enriched in stable microtubules with little dynamicity, on the contrary tyr-microtubules appear to be very dynamic (Kreis, 1987). Moreover, evidences show that all interphase microtubules are initially tyrosinated and daughter cells, immediately after mitosis, have mostly only tyrosinated microtubules (Kreis, 1987). The detyrosination occurs later on polymeric tubulin.

The monoclonal anti-tyrosine tubulin reacts against tubulin´s C-terminal tyrosine in immunoblotting assay. A peptide containing the carboxy terminal amino acid of alfa-tubulin was in fact used as immunogen (from the product sheet). Therefore, it has been used for localizing the tyrosinated α-tubulin in the brain and visualizing the main structure of the niche and the stream which are apparently very rich in tubulin. Reactivity has been reported for plant, animal, human, yeast and *Xenopus* (from the product sheet).

6.1.6.3. Monoclonal Anti-engrailed/invected gene products (1:25, Developmental Studies Hybridoma Bank, 4D9)

The generation of the monoclonal antibody 4D9 has been described in detail by Patel and colleagues (1989). The immunogen used were the C-terminal two-thirds of the *Drosophila melanogaster invected* protein as generated in *Escherichia coli* with the T7 polymerase expression system. BALB/c mice were immunized with the proteins by intra-peritonial injections. Their spleen cells were fused with NS-1 myeloma cells (Patel et al.

1989). Antibody 4D9 is an IgG1 that recognizes the *engrailed* and *invected* gene products of *D. melanogaster*. The epitope has been localized to residues 38–58 of the homeodomain, and this epitope is conserved between Invected and Engrailed proteins. Immunolocalisation experiments with this antibody in *D. melanogaster*, grasshoppers, crayfish and other crustaceans have provided evidence for consistent staining patterns during embryonic segmentation, suggesting an evolutionary conservation of the epitope that 4D9 recognizes across the Euarthropoda (Patel et al. 1989; Scholtz et al. 1993, 1994; Harzsch 2003b; Alwes and Scholtz 2006). Furthermore, the individual identification of Engrailed expressing (En+) neurons with 4D9 and a comparison of these neuronal staining patterns across several insect and crustacean species strongly suggested that 4D9 binds in a pan-specific way (Duman-Scheel and Patel 1999). Abzhanov and Kaufman (2000) have cloned partial cDNAs of *engrailed* from crayfish and for this organism have reported the presence of two *engrailed* paralogous, *en1* and *en2*, similar to the situation in insects. By comparing *in situ* hybridization expression data with the 4D9 immunolocalisation patterns, Abzhanov and Kaufman (2000) concluded that monoclonal antibody 4D9 in crustaceans recognizes both *engrailed* paralogous.

6.1.7. Proliferation markers (BrdU and EdU)

Proliferation markers such as BrdU (5-Bromo-2-deoxyuridine) and EdU (5-ethynyl-2'-deoxyuridine) were used in order to identify cells in the S-phase of the cell cycle which are going to divide. They are usually employed to estimate the mitotic activity of cells. More details will be given afterward in this chapter.

6.1.8. Secondary antibodies

According to the type of experiment, different cocktails of secondary antibodies were used. In singly labeled preparations, primary antibodies were detected with goat anti-mouse Alexa 488, goat anti-rabbit Alexa 488, goat anti-rat Alexa 488, goat anti-rabbit Alexa 546, goat anti-mouse 546, goat anti-rat 546 and secondary antibodies (1:50; Invitrogen). In double-labeled preparations, CY3 anti-rabbit, CY3 anti-mouse were also used (1:50; Jackson Immuno Research).

6.1.9. Counterstaining

Phallodin and nuclei counterstaining were used not only to contrast the principal labeling by adding a further source of color to samples, but also to provide more details of the structure of the brain. As reported in the product sheet, Phalloidin is a toxin, isolated from the deadly *Amanita phalloides* mushroom, which binds to actin. Consequently, labeled phallo-toxins such as Alexa 488-phalloidin (N. A12379, Invitrogen) and Alexa 546 phalloidin (N. A12380, Invitrogen) represent convenient probes for labeling and identifying F-actin in tissue (1:50). Unlike antibodies, the binding affinity is more stable because it does not change with actin from different species (from the product sheet). By using phalloidin, emerging structures of the brain during embryogenesis were highlighted and provided a valid background for determining the exact developmental stages of specimens. As nucleic acid stain, the green fluorescent YOYO (0,25 µl/ml; Invitrogen; NoY3601) was used in order to distinguish nuclei (hence cells) from acellular structure of the brain (i.e. the central pore of the niche, Chapter 5).

6.1.10. Basic procedures

- *Reagents for immunohistochemistry*

Solution A (Sol A): 33,9g Na_2HPO_4 in 1000ml distilled water.

Solution B (SolB): 25,9g K_2HPO_4 in 1000ml distilled water.

PBS (phosphate buffer 0,1 M, pH 7.4)

1000ml: 400ml Sol A + 100ml Sol B + 500ml distilled water + 5,80g NaCl + 200µl TritonX-100. Check/adjust pH to 7.2-7.4; store at RT. For use as wash/storage medium add 0.1% NaN_3 (sodium azide) (=0.1g/100ml).

PBS-TX (0,3% Triton X, Na-Azid 1% BSA)

10ml PBS + 30µl Triton X + 100mg BSA + 100µl NaN_3 (5% stock solution).

PFA (4% paraformaldehyde): add 4 gr PA (paraformaldehyde) in 50 ml of distilled water, then heat at max. 60 C (use specific thermometer), add few drops of NaOH to the solution until all PFA has been dissolved. Add 40ml of PBS and 10ml of Sol B. Filter and distribute in different aliquots of 5 or 10ml. Store in the freezer for 2 months. Note: work under fumehood.

PEM-FA

0.1Mpipes buffer pH 6.95, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde

PTw (1 liter)

100ml 10XPBS, 5ml 20% Tween-100, 895ml RNAse-free water. Store at room temperature.

PBT (1 liter)

990ml 1XPBS, 10ml 20% Tween-100, 1gr BSA. Store in the fridge.

- *Reagents and media for molecular biology*

All commonly used buffers, solutions and bacteria media for the molecular analysis were prepared as described in Sambrook (2000) and solutions and media were sterilized by autoclaving.

Luria-Bertani (LB) Medium (1 litre) (for bacteria)

- 10g tryptone
- 5g yeast extract
- 10g NaCl
- double distilled water added to 1 litre and adjusted to pH 7.5

After cooling media containing agar or agarose, thermolabile substances such as antibiotics, IPTG and X-Gal were added. Media were then poured to 90mm petri dishes and left to dry for about 20 minutes at room temperature. Plates were used either immediately or stored at 4°C for up to a month. Ampicillin plates were prepared by adding ampicillin to a final concentration of 100μg/ml to the medium. Ampicillin was stored as stock solution at 100mg/ml at –20°C.

20 X SSC (1 liter)

175.3g NaCl (3M), 88.2g Na Citrate (0.3M), pH 4.5. Autoclave.

Hyb Buffer (40ml)

Formamide (50%; Roche) 20ml; 10ml 20 X SSC, 0.1 Heparine (20mg/ml), 0.5ml 20% Tween-100, 2ml 20% SDS, 0.2ml salmon sperm DNA (10mg/ml; Sigma), 0.1mg/ml tRNA (Sigma), 7.5ml distilled water.

Blocking Buffer (10X)

5ml 10% Blocking Reagent (powder), 45ml 1X Maleic Acid Buffer (90%)

Maleic acid Buffer (10X; 1 liter)

116g Maleic Acid, 88g NaCl, milliQ up to 1 liter, 7g solid NaOH, pH 7.5. Autoclave.

Alkaline Phosphate Buffer (50ml)

5ml 1M NaCl, 2.5ml 1M MgCl2, 5ml 1M Tris pH 9.5, 1.25ml 20% Tween-20, 36.25ml distilled water.

Alkaline Phosphate substrate solution (2ml)

2ml Alkaline Phosphate Buffer, 35.2µl NBT/BCIP (18.75/9.4mg/ml). Keep in the dark.

NBT/BCIP substrate mixture: 4-Nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolylphosphate (Roche).

6.1.11. Standard immunohistochemical protocol

Crayfish embryos of different developmental stage were dissected in crayfish saline (mM: 205 NaCl, 5.4 KCl, 34.4 CaCl2, 1.2 MgCl2, 2.4 NaHCO3, pH 7.4). Brains were removed from the embryos and either fixed for 4 hours at room temperature (rt) or overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer. Subsequently, brains were rinsed for 2 hours in PBS, and incubated for half an hour PBS-TX, before the incubation in the primary antibodies (4 hours or overnight at 4°C). After incubation in the primary antibodies, specimens were rinsed for 2 hours in PBS, preincubated in PBS-TX and then incubated overnight in the appropriate secondary antibodies. Some brains were also counterstained with the nuclear stain YOYO (0,25 µl/ml) for 15 minutes. Afterwards, samples were rinsed for 2 hours in PBS and mounted either in Gelmount (Biomeda, Foster City, CA) or Mowiol (coverslip mounting solution of polyvinyl alcohol Mowiol 4.88, Calbiochem catalog number 475904).

6.1.12. Protocols for BrdU and Edu

6.1.12.1. BrdU (5-Bromo-2-deoxyuridine)

5-Bromo-2-deoxyuridine (BrdU) is a synthetic thymidine analog which is incorporated into single strand DNA during the S-phase of the cell cycle (Fig. 6. 5). It can be used to monitor the proliferation of cells in living tissues. Live hatchlings or juveniles' crayfish

were incubated directly in the BrdU solution (0,2mg BrdU/ml, cell proliferation kit Amersham RPN 201), animals were incubated in a saline solution after removing the chorion, to improve the penetration of BrdU into the tissue. Incubation was for 4 hours at room temperature. Subsequently, animals were fixed in PFA for 30 min. at room temperature before dissection. Brains were removed and incubated in 4% paraformaldehyde (PFA) for at least 4 hours at room temperature or overnight at 4C. Specimens were washed in at least 3 changes of PBS buffer for 30 min. and then preincubated in PBS-TX for 30 min. To improve binding of the antibody, the tissue was exposed to an acid treatment (2N HCl) for 20 min. to denaturate the DNA structure. After another cycle of washing steps with PBS buffer, brains were incubated in primary anti-BrdU antiserum for 2.5 hours at rt (from rat or mouse, according to the experiment). Afterwards, specimens were washed in PBS overnight at 4° C and incubated in the secondary antibody, a goat anti-mouse antiserum conjugated with peroxidase, for 60–90 min at room temperature (rt). Specimens were washed in PBS overnight at 4° C. To detect the bound antibody, the enzymatic DAB (diaminobenzidine) reaction was used according to the manufacturer's instructions: in presence of cobalt and nickel, DAB gives a blue-black precipitation where the secondary antibody is bound and hence in those nuclei that incorporated BrdU. After dehydratation in EtOH, specimens were mounted in methyl salicylate.

6.1.12.2. EdU (5-ethynyl-2'-deoxyuridine)

Click-iT® EdU Cell Proliferation Assays (Molecular probes) is an alternative to the BrdU method and thus S phase specific. It is not antibody based and does not require DNA denaturation for detection of the incorporated nucleoside. EdU (5-ethynyl-2'-deoxyuridine) is a thymidine analog which is incorporated during the DNA synthesis. Detection is based on a click reaction,1-4 a copper catalyzed covalent reaction between an azide and an alkyne. In this case, the EdU contains the alkyne, while the Alexa Fluor® 488 dye contains the azide (Fig. 6. 5). Animals (embryos, hatchlings and juveniles) were incubated for 4 hours at room temperature and immediately fixed in PFA for 30 min. Dissection was performed after this first fixation and brains were fixed for 4 more hours afterward. Specimens were then washed in at least 3 changes of PBS buffer and pre-

treated in PBS-TX for 30 min. Incubation in Alexa 488 Click-it reaction cocktail was performed for 2 h at room temperature. The solution was prepared fresh and contained 1X reaction buffer, $CuSO_4$, AlexaFluor azide and Reaction buffer additive. Several washing steps followed this application before the incubation in primary antibodies for the double labeling, according to experiments.

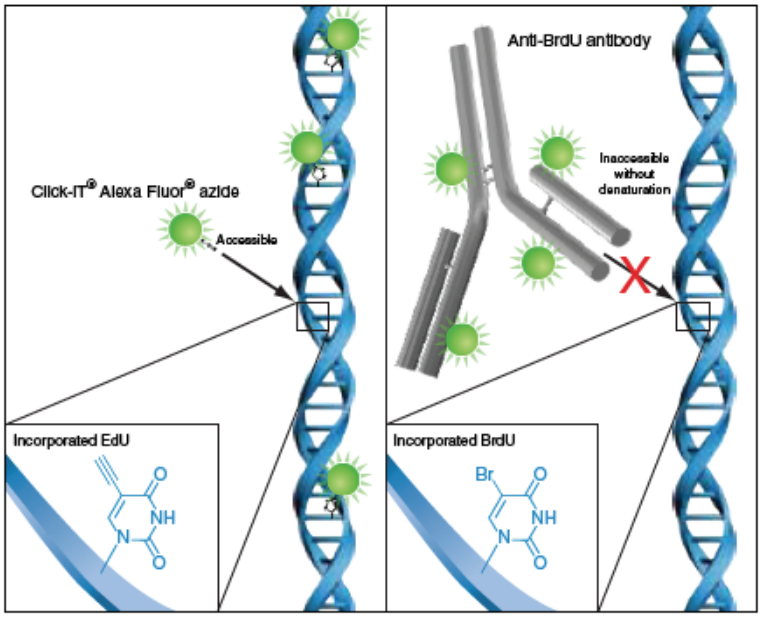


Fig. 6.5- Schematic diagrams of the principles of EdU (on the left) and BrdU (on the right) (from the EdU product sheet, Molecular Probes website).

6.1.13. BrdU-EdU pulse-chase experiment

In order to clarify the activity over time of cells residing in the niche, I performed a series of pulse-chase experiments by exposing animals to two the analogue proliferative S-phase markers (BrdU and EdU) at different times. Animals, placed in a small container for liquid (100 ml volume), were exposed first to BrdU for 4 hours (first pulse)(Fig. 6. 6). Then, the labeling solution was quickly rinsed off the animals and these were kept undisturbed in fresh water for 24 or 48 hours, depending on the experiment. During this time, animals were in similar rearing conditions as before the treatment, which means they were free to move, to interact with each other and to eat at the conditions as before the labelling procedure. After this survival period, animals were exposed to the second labeling reagent (EdU) for 4 hours (second pulse) and immediately fixed and dissected (Fig. 6. 6).

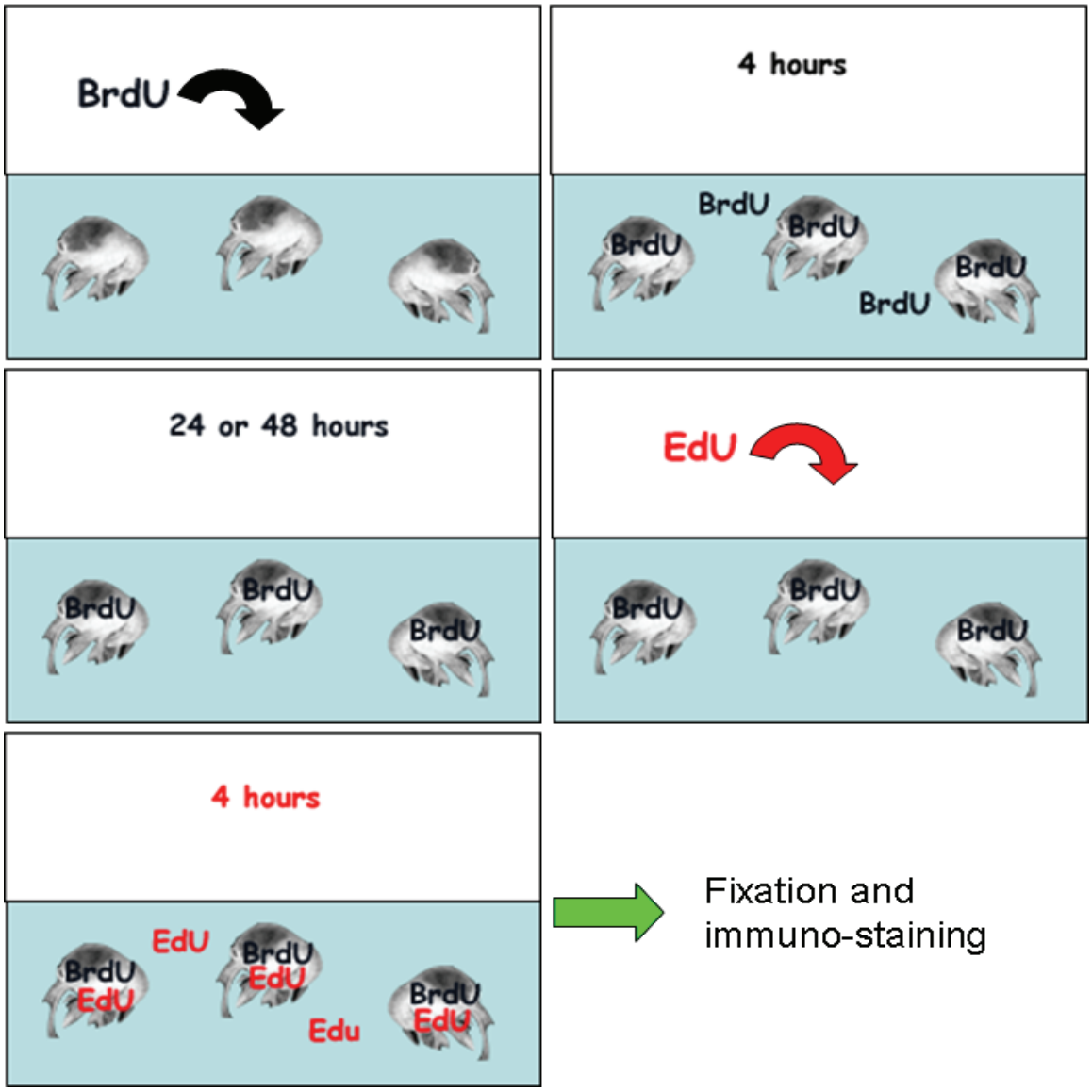


Fig. 6.6- Experimental design of the BrdU-EdU pulse-chase experiment applied to POI Marbled crayfish.

Protocols followed the same directions described above: several washing steps with PBS, acid treatment, incubation with primary anti-BrdU from rat (1:100 in PBS-TX), rinsing and incubation in primary anti-TyrosinTubulin from mouse (1:1000) overnight). The application of the Alexa 488 Click-it reaction cocktail was performed for 2 hours at room temperature. A cocktail of secondary antibodies made of Alexa 630 anti-mouse and Alexa 546 anti-rat was prepared.

After an incubation of 4 hours (or overnight at 4° C) and final washings, brains were mounted in Mowiol mounting medium.

6.1.14. Engrailed

Dissected animals were fixed in PEM-FA for 30 min at rt. After fixation, whole mount embryos were washed in at least three changes of PBS buffer for 30 min and pre-incubated in PBS-TX for 30 min. We used the primary monoclonal anti-Engrailed 4D9 (Patel et al. 1989), supplied as partially purified IgG (166 μg/ml) by the Developmental Studies Hybridoma Bank (University of Iowa). The antiserum was diluted 1:25 in PBS-TX and incubation was performed overnight at 4°C. Embryos were then washed several times in PBS for 1 1/2 h and incubated in a peroxidase-conjugated anti-mouse antibody (Sigma) (1:1,000 in PBS) for 2 h at room temperature. Afterwards, specimens were washed in several changes of PBS and kept in buffer overnight. The following day, the tissues were incubated for 2–3 min in a solution of 0.025% diaminobenzidine (DAB), to which a few drops of metal enhancer (3% H_2O_2/$NiCl_2$) were added to intensify and increase the contrast of the stain (Amersham BrdU kit RPN 20). After the reaction, specimens were washed for at least 4 h in PBS, washed in distilled water for 10 min and then dehydrated in increasing concentrations of ethanol up to 100%. Finally, specimens were cleared in methyl salicylate for 20 min and mounted in Entellan (ProSciTech, N. IM022) diluted in methyl salicylate. Around 50 embryos were processed in this way.

6.1.15. Double staining for Engrailed and actin

Dissected animals were fixed in PEM-FA for 30 min at room temperature. After fixation, embryos were washed in at least three changes (30 min) of PBS and pre-incubated in PBS-TX for further 30 min. Incubation in the primary anti-Engrailed antibody (4D9), diluted 1:25 in PBS-TX, was performed overnight at 4°C. Specimens were then washed in several changes of PBS for 1 1/2 h. Finally, they were incubated in a mixture of a secondary anti-mouse Alexa 488 antiserum and Alexa 546 phalloidin in PBS-TX for 1 h at room temperature. Afterwards, embryos were washed in several changes of PBS for about 1 h and mounted in Gel Mount (Sigma). Around 20 embryos were processed in this way.

6.1.16. Microscopic analysis and size measurement

Bright field digital images of the En+ cells in the brain were obtained by a Zeiss Axioskop fitted with a CCD-1300B digital camera (Vosskühler) and processed with the

Lucia Measurement 5.0 software package (Laboratory Imaging). Fluorescent samples were screened with a Zeiss LSM 510 Meta confocal microscope. Size measurements of labeled cells were taken using the tools of the Lucia Measurement 5.0 software package (Laboratory Imaging). By drawing the image cell borderline on the computer screen, it was possible to calculate the cell area. Drawings of the specimens were obtained by a *camera lucida* device at an additional 11X magnification. Measurement of cells residing the niche were obtained by using the software ImageJ. Size of images in two dimensions was 1024 X 1024 X 1 pixel, sizes of images in three dimensions (z stack) varied according to the sample. Images from stacks represent projection of the z sections.

6.1.17. Cloning

In order to study the primary and secondary structure and the expression pattern of *Pax6*, *atonal* and *orthodenticle* during embryogenesis, cloning the genes into appropriate vector was the first step to perform. By using the appropriate primers, PCR (Polymerase Chain Reaction) was run on the cDna (complementary DNA) deriving from the mRNA of embryos at about 45% of embryonic developmental stage. Once the DNA of interest was isolated from the total cDNA, a ligation procedure was applied to insert the amplified fragment into a vector (usually a plasmid, a double-stranded circular DNA able to replicate in a host cell). The vector was linearised using specific restriction enzymes, and incubated with the isolated fragment and the enzyme DNA ligase. After ligation, the vector with the insert of interest was transfected into cells by heat-shock treatment that allows the entrance of the vector into the cell. Finally, the cells were cultured. To identify cells that were successfully transfected with the plasmid, a screening method was applied. Usually cloning vectors include antibiotic resistance markers which allow the growth only to cells in which the vector has been transfected. Moreover, the cloning vectors may contain color selection markers which provide blue/white screening on X-gal medium. Nevertheless, these screenings do not guarantee that the DNA insert is present.
So, usually, a colony PCR and DNA sequencing were applied to confirm the sequence.

6.1.17.1. Extraction of Total RNA

In order to apply molecular techniques such as cloning and *in situ* hybridization, an intact RNA is a crucial step to achieve because ribonucleases in the tissues can rapidly degrade

RNA. One of the most common methods to extract RNA is the Guanidinium thiocyanate-phenol-chloroform method, usually called Trizol. It functions by preserving the RNA integrity during homogenization of the tissue, and by disrupting tissue at the same time.
Total RNA was isolated from embryos (whole egg) of early stages (around 45%) by using the Trizol Method. Eggs were frozen by liquid nitrogen and smashed into powder. About 1 mg of frozen powder was placed in Rnase-free Eppi tube. Tritol was added and mixed with the tip for 5 min at room temperature until complete dilution. The protocol is the following:

- add 200 µl Chloroform under the hood
- shake by hand for 15 sec
- leave it on the rack for 2 min at room temperature
- centrifuge at 13,500rpm (max) for 15 min at 4°C
- recover the supernatant (aqueous phase which corresponds to the RNA)
- pour it in a new Eppi RNAse-free tube
- add 500µl Isopropanol
- shake it by hand
- leave it on the rack for 10 min at room temperature
- centrifuge at 13,500rpm (max) for 10 min at 4°C
- remove the supernatant
- add 1ml 75% RNAse-free Ethanol
- Vortex to dissolve the pellet of RNA
- centrifuge at 7,500rpm (max) for 5 min at 4°C
- remove the supernatant
- centrifuge at 7,500rpm (max) for 1 min at 4°C
- remove all the Ethanol (dry on the paper)
- leave tube opened in the rack for 5 min at room temperature
- add 50µl RNAse-free ddH_20
- dissolve by pipetting
- incubate In water bath at 55°C 2X5 min
- if not dissolved: add 1ml RNAse-free ddH20
- Incubate in water bath at 55°C then dissolve by pipetting

- check RNA isolation using Nanodrop spectrophotometer (1µl)
- chill on ice or store at – 80°C.

6.1.17.2. The reverse transcritption

This step allows the synthesis of cDNA (complementary DNA) from mRNA which can be separated by the rest of total RNA by using oligomeric dT nucleotide coated resins where only the mRNA with the poly-A tail will bind.

The protocol is the following:

- add 1-2µg of total RNA in a RNAse-free tube
- add 2µl OligodT
- add 12µl RNAse-free water
- mix and spin briefly
- heat for 3 min at 70-85°C
- put the tube on ice
- spin briefly
- put the tube on ice
- add the remaining components:
- 2µl 10X RT Buffer
- 4µl dNTP mix
- 1µl RNAse inhibitor
- 1µl Reverse Transcriptase M-MLV
- 20µl is the final volume
- mix gently
- spin briefly
- incubate in water bath at 42-44°C for 1 hour
- incubate at 92°C for 10 min to inactivate the reverse transcriptase
- spin briefly
- store at -20°C or proceed to PCR reaction.

6.1.17.3. Computer Programs

Computer softwares and Internet resources were used for this project and run on a Macintosh G3 (Tab. 6.1).

Programs	Use
WordMac	Text program
Sequencher 3.0	Sequence assembly
MacVector	Primer design
MEGA	Sequence alignment
Internet Resources	
BLAST	Non-redundant protein database search
Boxshade 3.21	Multiple alignment design
NEBcutter V2.0	Unique restriction enzymes sites

Tab. 6.1- Computer softwares and Internet resources used for the project.

6.1.17.4. Bacterial strains

Different strains of *Escherichia coli* (DH5α, NM522) were used for transformation of plasmid vectors. Bacterial strains were made Ca^{2+}-competent (see below), and snap frozen in liquid nitrogen, before being stored at -70°C as described in Sambrook (2000).

6.1.17.5. Plasmid Vector

pGEM-T vector (Promega) was used for routine cloning procedures. The general characteristics are reported in Fig. 6.7.

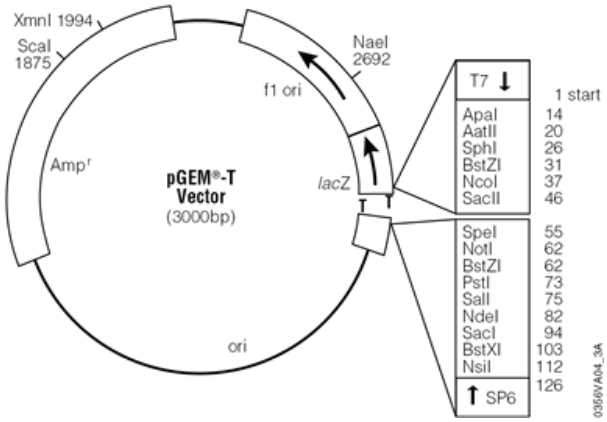


Fig. 6.7- pGEMT-T (Promega) vector structure and characteristic.

6.1.18. Basic procedures

Some frequently used methodologies are described here and only modifications are reported later if necessary.

- *Agarose-gel Electrophoresis*

Agarose gel electrophoresis was performed in Bio-Rad Electrophoresis tanks, using 1XTAE Buffer. Usually, 1% gels were run at 87V for 40-45 minutes at room temperature. Ethidium bromide was added to the agarose gel before the gel was poured. Orange G Sucrose Loading Dye was added to samples before loading. Ethidium bromide was visualised using a UV transilluminator.

- *Polymerase-Chain-Reaction (PCR)*

PCR was used for a variety of applications:

- amplifying target sequences in cloning procedures
- to insert cutting sites for cloning into expression vectors
- to check if insert of choice was present in the plasmid of a bacterial colony

A typical PCR reaction consisted of:

- 2µl DNA template (100ng)
- 1,5µl Forward primer (20µM)
- 1,5µl Reverse primer (20µM)
- 3µl dNTPs (2mM each)
- 3µl 10X DNA Polymerase Buffer (MgCl included)
- 0.3µl Taq polymerase (5U/µl)
- Nuclease-free H_2O to 30µl

The typical thermal cycling conditions for the amplification of DNA fragments by PCR are shown in Tab. 6.2.

Step	**Temperature**	**Time**	**Number of cycles**
Denaturation	95°C	30 seconds	35
Annealing	Ta*	30 seconds	35
Extension	72°C	30 seconds	35
Final extension	72°C	2+(1minute/kb)	1
Hold	4°C	holding step 24 hours	1

Tab 6.2- Typical thermal PCR cycling conditions. * Annealing temperature (Ta) was determined as 2-5°C below the melting temperature (Tm) of the primers.

- *Digestion*

All digestions were performed as described in (Sambrook, 2000) using Promega restriction enzymes and buffers. Reactions were performed for between 2 and 8 hours at the appropriate temperature, typically 37°C. In the case of multiple digests using enzymes requiring different buffering conditions, the digested products were precipitated and resuspended between successive digestions.

- *Ligations*

Ligations were performed as described by (Sambrook, 2000) using Promega T4 DNA Ligase and 10x Ligase Buffer, using a 1:1 molar ratio of plasmid vector (pGEM-T Easy): insert DNA. The reactions were incubated at 4^0C overnight or 25°C for 1-6 hours and the entire ligation reaction (10μL) used for transformation of competent cells.

- *Dephosphorylation of Cut Vector*

The method for dephosphorylation of the ends of a blunt end cut vector is modified from Sambrook *et al.,* (2000), as follows:

- eg. 19μL of digested vector
- 161μL of ddH_2O and 20μL Calf Intestinal Alkaline Phosphatase (CIAP)
- Buffer (supplied by Promega) was added,
- and 20μL was removed and retained as the non dephosphorylated vector control. 5μL (0.5U) CIAP enzyme was added to the remaining 180μL and incubated at 37^0C for 60 minutes.
- The reaction was then heated to 75^0C for 10 minutes with 5mM EDTA.
- ddH_2O was added to a volume of 800μL and the DNA phenol/chloroform extracted once, followed by one chloroform extraction.
- The solution was precipitated with 0.7 volumes of ice-cold isopropanol and NaOAc (3M, pH 5.2) to a final concentration of 0.3M.
- The DNA was allowed to precipitate at -20^0 C for 30 mins, and then spun at 13200 rpm for 20 minutes.
- The supernatant was removed, and the pellet washed in 1mL of 70% ethanol.
- The solution was then spun at 13,200rpm for 5 minutes, the supernatant removed again, and the pellet dried in a vacuum centrifuge.

- The DNA was resuspended in TE buffer (10mM Tris.HCl pH8.0; 1mM EDTA) to a concentration of 0.1μg/μL and stored at 4^0C.

- *Recovery of DNA from Agarose Gels*

DNA was recovered from Agarose Gels by filtration through Whatman Filter Paper. The Whatman paper was cut to a circular size of approximately 5cm in diameter and made into a cone shape by folding the paper around a 2 -20μL pipette tip which had the tapered end cut off. This was placed inside a 500μL PCR tube which had a needle hole pierced in the bottom. The filter paper/PCR tube contraption was then placed inside a 1.5mL microfuge tube and the agarose gel slice placed in the filter paper and immediately spun at 5000rpm for 5 minutes in a microcentrifuge. The eluate was then used in further experiments.

- *Ethanol Precipitation of DNA*

Precipitation of DNA allows for resuspension in a smaller volume and also removes residual chloroform which can inhibit many enzymatic reactions.

The procedure as adapted from Barker, 1998 is as follows:

To a maximum volume of 450μL of DNA in water/TE buffer, 1/10 volume of 3M NaAc pH 4.8 was added and inverted briefly to mix. Two volumes of 95% or 100% ethanol were added and the solution was mixed by inversion.

The DNA was precipitated by placing the sample in the cold at -20^0C overnight. The sample was then centrifuged at high speed (>12000rpm) for 15-30 minutes at 4^0C and the supernatant decanted. Tubes were drained by inverting and blotting with a paper towel. The pellet was washed with cold 70% EtOH and dried with the use of a vacuum centrifuge and allowed to resuspend in TE buffer pH 8.0 overnight at 4°C.

- *DNA Sequencing*

DNA sequencing was done in a thermocycler using the DYEnamic™ ET Dye Terminator Sequencing Kit from Amersham Biosciences. Four different fluorescent dyes are used to discriminate between the dideoxynucleotide A-, C-, G- and T- terminators during the reading of the sequence. The reactions were analysed on a MegaBACE™ 1000 DNA Analysis System (Amersham Biosciences) via a laser induced fluorescence capillary system.

The sequences were manually checked and assembled with Sequencer Version 3.1or 4.0. The cycle sequencing protocol for the DYEnamic™ ET Dye kit is as follows:

96°C 10 seconds
50°C 5 seconds } Cycles were repeated 25 times
60°C 4 minutes

- *DNA Quantification*

DNA quantification was determined spectrophotometrically using the Nanodrop spectrophotometer (1µl).

- *Competent cells*

The following preparation of Ca^{2+} competent E. *coli* is modified from Sambrook *et al*. (2000).

The chosen bacteria strain (DH5α or NM522) was picked from a cell stock frozen at –70°C, with a sterilized wire loop and streaked into LB agar plates. Between each set of parallel streaks the loop was flamed and the colony streaked again in parallel streaks, crossing the previous set. With this method, colony number was progressively reduced so that it was possible to get single colonies. Plates were then incubated overnight at 37°C.

The next day, a single colony was picked from the plate and inoculated into 5mLs LB broth and incubated at 37°C with moderate agitation overnight. From this overnight culture, 1mL was inoculated into 50mLs LB in a 500mL flask, and incubated at 37°C until cells reached a growth corresponding to a density of between A_{600} 0.4-0.6.

The bacterial culture was placed on ice and cooled to 0^oC. The mixture was then transferred to a 50mL ice-cold Falcon Tube and spun at 3500rpm for 10minutes at 4^oC. The supernatant was drained well and the pellet retained. 10mL ice-cold 0.1M $CaCl_2$ solution was added to the pellet, and the bacteria gently resuspended over 1 hour on ice. Cells were recovered by centrifugation at 4000rpm for 10minutes at 4^0C. Again the supernatant was drained and the pellet retained. The pellet was then resuspended in 2mL ice-cold 0.1M $CaCl_2$ over 1 hour on ice. Glycerol was added to a final concentration of 20%, and the resulting solution dispensed into aliquots and snap-frozen in Liquid Nitrogen. The competent cells were stored at $–70^0C$.

- *Tranformation of competent cells*

The transformation of competent cells was carried out following the protocol of the heat shock method described in Sambrock (2000).

An aliquot of competent cells was taken from the –70°C freezer and put on ice. Between 50 and 100µl of cells was added to 10ng of circular DNA directly (or 10µL of a ligation reaction) and incubated on ice for 20 minutes. After this period of time, the suspension was heat shocked for 2 minutes at exactly 42°C. The cells were then cooled back on ice for 10 minutes and 1ml of LB medium was added to the tube. Transformed cells were then allowed to grow at 37°C for one hour in a shaking incubator at approximately 200rpm. Cells were then recovered by centrifugation at 8000 rpm for 15 seconds in a tabletop centrifuge. Part of the supernatant was discarded (about 800µl) and the remainder used to resuspend the cells. The suspension was then plated on LB Agar medium with the appropriate antibiotic to allow selection of transformants.

- *Colony PCR*

A colony was picked off a transformed plate with a sterile toothpick and resuspended in 3µL dH_2O. The solution was then incubated at 95^0C for 5 minutes, centrifuged and placed on ice before being used in a typical PCR reaction with primers specific to the insert of interest or primers which annealed to vector sequence surrounding the insert. After the PCR was complete, the samples were run on an agarose gel, and positive colonies identified by the presence of bands on the gel which indicated amplification of the insert had taken place. The colonies which were positive for the insert of interest were then used in further manipulations.

- *Isolation of plasmid DNA (Minipreps)*

In general, plasmid DNA was extracted using QIAGEN or BIORAD Miniprep Spin Kits which gave typical yields of approximately 100ng/µL of plasmid DNA.

- *Oligonucleotide Primer Design*

Primers for use in routine experimental procedures such as PCR and sequencing reactions were designed using MacVector™ with parameters limiting the length to 18–25 nucleotides, the melting temperature to 55-60°C and the G/C content to 45–55%. Oligonucleotide primers were supplied by Geneworks and were stored as 100µM or 200µM stock solutions at –20°C for dilution and use in reactions.

The following primers were used for cloning *Pax6*, *atonal* (D1, D2), *otd* and *ems*:

Primer name	**Sequence**	**Length**	**Tm C**
Pax6 1	CAYWSNGGNGTNAAYCARYTNGGNGG	26	57
Pax6 2	AACCANACYTGDATNCKNGCYTCNGG	26	56
Pax6 3 (nested PCR)	ACNMGNCARAARATHGTNGARYTNGC	26	53
Pax6 4 (nested PCR)	TARTGNGTNCKYTCRAAYTCYTTYTC	26	51
otd1	MGNGARMGNCANCANTTYAC	20	47
otd2	GCNCARYTNGAYGTNYTNGA	20	47
otd3	CKRTTYTTRAACCANACYTG	20	43
otd4	TTNGCNCKNCKRTTYTTRAACC	22	48
otd5	YTGYTGNCKRCAYTTNGC	18	44
ems1	AARCCNAARMGNATHMGNAC	20	44
ems2	CANGCNTTYWSNCCNWSNCA	20	49
ems3	CCANACYTTNACYTGNGTYTC	21	47
D1	IATRTAIGTYTGIGCCATM	19	38
D2	GCIGCIAAYGCIMGIGARMG	20	49

Tab. 6.3- Primers required for the construction of Pax6-otd-ems and atonal (D1, D2) –pGEMT easy vector.

6.1.19. Phylogenetic Analysis

The cladograms were constructed using the MEGA program (Molecular Evolutionary Genetics Analysis; Kumar et al., 2008). The aminoacid sequences of known species were downloaded from GenBank and aligned in FASTA format by using CLUSTAW. The reliability of the trees was estimated by Bootstrapping, one of the most commonly used tests (Felsenstein, 1985). Bootstrapping is indicated in percentage and represents the percentage of cases in which all species derived from that node appear together in the

trees. Bootstrap values above 90% are statistically strongly reliable; below 70% are considered unreliable.

6.1.20. *in situ* hybridization

6.1.20.1. The probe

Different types of probes can be used in *in situ* hybridization. The single strand RNA probe is considered a good choice because of the RNA-RNA interactions that are more stable than DNA-RNA hybrids. This allows stronger washing conditions which provide a better signal. Moreover, RNases can enzymatically destroy unhybridized RNA probe, and this reduces the background. One disadvantage with RNA probes is the higher level of non-specific binding to tissue components, which may cause higher background and lower penetration of the probe into the tissue (Jin and Lloyd, 1997).

6.1.20.2. Generation of the Pax6 - Digoxigenin - probe

Probes can be synthesized using different protocols. Basically, the generation of RNA probes is based on in vitro transcription of cDNA by incorporating modified nucleotides. The RNA probe is transcribed from a linear DNA template using highly specific bacteriophage DNA-dependant RNA polymerases from the *Salmonella bacteriophage* SP6, and the *E. coli* bacteriophages T3 and T7 (RNA polymerase T7, T3 or SP6). For *in vitro* transcription reactions restriction enzymes are used to linearize the plasmid (DeBault and Gu, 1996). Riboprobe vectors, like pGEM-T (the one I used), have the RNA polymerase initiation sites near unique restriction sites used to clone the chosen cDNA in. Depending on the orientation of the cDNA insert in the riboprobe vector, relative to the SP6 or T7 sites, either “sense” or “antisense” RNA probes can be produced. Transcription from the cDNA template using SP6 RNA polymerase produces a nucleotide sequence identical to the mRNA, the so called “sense” transcript. Since the “sense” probe is the same sequence as the cellular mRNA it will not bind during the hybridization reaction and so it can be used as negative control. Transcription of the cDNA template from the opposite side (using the T7 RNA polymerase initiation site) will

synthesize an "antisense" RNA probe which will hybridize to the mRNA. Transcription occurs in the presence of a labeled nucleotide (DeBault and Gu, 1996; Fig. 6. 8).

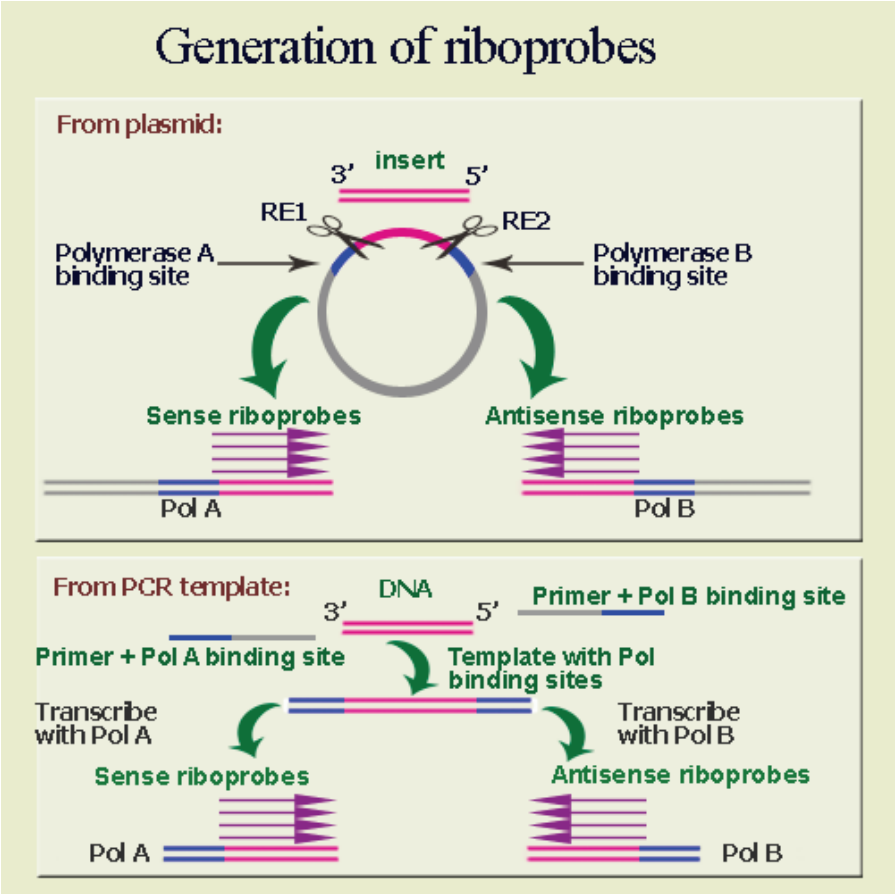


Fig. 6.8- Scheme of the main steps of the generation of a riboprobe, both from a plasmid and from a PCR template.
(from the NCBI website: http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechISH.shtml).

One of the most common methods to label the probe is based on Digoxigenin-labeled probe. Digoxigenin (DIG) is found in flowers and leaves of the plant *Digitalis purpurea*, *Digitalis orientalis* and *Digitalis lanata* (Polya, 2003). It is used as immunohistochemical marker for *in situ* hybridization. It is conjugated to a nucleotide (usually uridine), and then incorporated into a riboprobe. Later, the digoxigenin markers are detected with anti-digoxigenin antibody. The DIG RNA Labeling Kit (Roche) was used for this purpose. In this method, the vector in which the sequence is cloned contains phage transcription promoters (SP6 and T7 promoters) that can initiate the transcription in the presence of the corresponding RNA polymerase. Digoxigenin (DIG)-labeled UTP is supplied and is

incorporated into the transcript, generating DIG-labeled RNA. The first step of the protocol is to linearize the template (Tab. 6.4). In order to do so, the endonucleases ApaI and SpeI were used respectively for opening the plasmid and generating the sense probe (the control) and the antisense probe. The restriction enzymes were chosen based on the unique sites present in the vector and not within the insert. The software Nebcutter was used for this operation.

In RNase-free tubes, the following elements were added:

SpeI (antisense probe)	**ApaI (sense probe)**	**Control**
11µl H_20	11µl H_20	11µl H_20
2µl Buffer	2µl Buffer	
2µl SpeI	2µl ApaI	
6µl DNA (from Miniprep)	6µl DNA (from Miniprep)	6µl DNA (from Miniprep)
Final Volume: 20µl	Final Volume: 20µl	

Tab 6.4 – Linearization of the plasmid by digestion with endonucleases

After checking the enzymes have produced the right band, the endonucleases are removed by precipitation before transcription. Transcription by labeled nucleotides was performed as follows:

- 1µg linearized template
- 13µl diethylpyrocarbonate (DEPC) treated water
- 2µl dNTP labeling mixture
- 2µl transcription buffer
- 1µl RNase inhibitor
- 2µl of the appropriate SP6 or T7 RNA polymerase
- then incubate for 2 h at 37ºC
- to stop the reaction, add 2 ul of 0.2 M EDTA.

To visualize the Digoxigenin incorporated into the riboprobe, two methods were used:

- The first one based on the Anti-Digoxigenin-AP, Fab fragments kit (Cat. No. 11 093 274 910 150, Roche) in which Fab fragments, from a digested anti-digoxigenin antibody from sheep conjugated with alkaline phosphatase (**AP**), bind to digoxigenin. The Alkaline phosphatase activity is visualized with 5-bromo-4-chloro-3-indolyl-phosphate (**BCIP**) and nitro-blue-tetrazolium chloride (**NBT**), which produce a black-purple precipitate. The sensitivity and precision of alkaline phosphatase-NBT/BCIP staining is very high and is often the preferred method over fluorescent *in situ* techniques.

- The second method consisted in recognizing the probes with fluorescent antibodies (FISH, Fig. 6. 9) and for this purpose the HNPP Fluorescent Detection Set was used (Cat. No. 11 758 888 001, Roche).

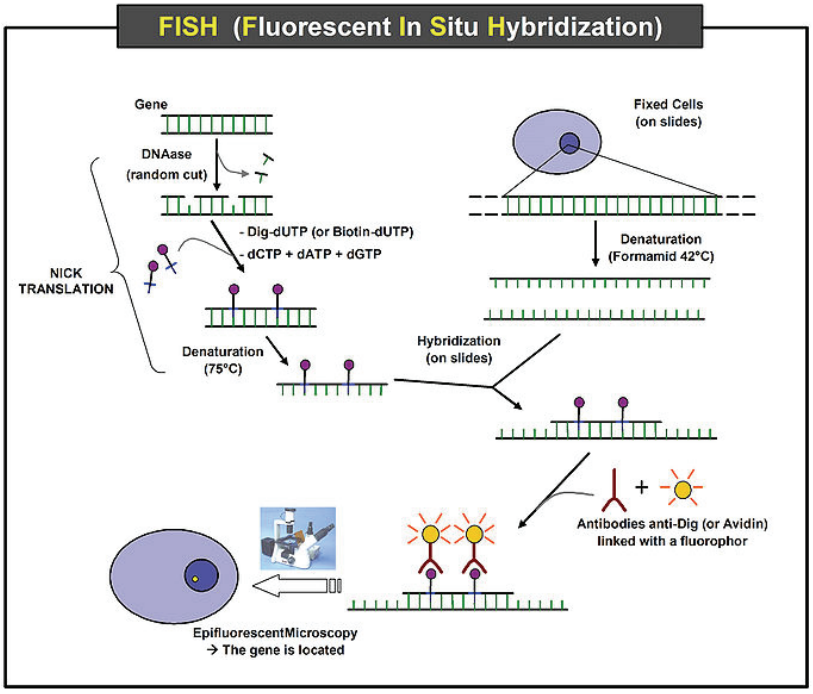


Fig. 6.9- Main steps of the FISH technique to localize a gene in the nucleus (image from Wikipedia).

HNPP (2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate) is a fluorescent substrate for alkaline phosphatase. As HNP (dephosphorylated HNPP) has a weak affinity to tissues, upon dephosphorylation, HNPP produces a strongly fluorescent precipitate on membranes with a maximum of absorbance at 290 nm and a maximum of fluorescence emission at 509 nm" (from Roche product description sheet). For *in situ* applications HNPP is coupled with the diazonium salt Fast Red TR to increase the affinity to tissues

and to ensure precipitation of the dephosphorylated form (HNP) *in situ*. The reaction product generated after dephosphorylation (HNP/Fast Red TR) is a highly fluorescent precipitate that accumulates during the reaction. The absorbance maximum of combined HNP/Fast Red TR precipitate is shifted towards 553 nm and a broad fluorescence emission (between 565–620 nm) with a maximum at 584 nm is generated (from Roche product website).

6.1.21. *in situ* protocols in Marbled crayfish

Various protocols were tested on embryos of different developmental stage. The majority of them were simply modification of standard ones, reported below.

The material used for *in situ* hybridization can be fixed in a number of ways, according to the type of tissue or analyses to be performed.

Here some of the methods are reported:

- specimens can be fixed in paraformaldehyde (PFA), as one would normally fix tissues for histology, and used whole mount
- or embedded in wax and sectioned it with the Vibratome
- or can be snap frozen and embedded in a specific support medium for cryosectioning. The sections are then quickly fixed in paraformaldehyde.
- tissues can be fixed and dehydrated in methanol.

In my case, fixation was performed with PFA at different concentration. Fixation times changed between 1 and 4 hours.

Protocols 1-2-3 are NBT/BCIP based**,** Protocol 4 is a FISH method.

Protocol 1

(based on Prpric work on Amphipod. Personal comunication)

Fixation: 4% paraformaldehyde. After initial fixation, the yolk was removed and the embryos were dehydrated in a methanol series and stored in 100% methanol at -20C.

DAY 1

Pretreatment

Transfer embryos to eppendorfs (1 for the antisense probe, one for the sense probe), use about 900-1000µl for each wash

- Rehydrate:

Wash 5 min in 75% MeOH/ 25% PTw

Wash 5 min in 50% MeOH/ 50% PTw

Wash 5 min in 25% MeOH/ 75% PTw

- Wash 3x5 min in PTw
- proteinase K, make a dilution of 1:10 from the stock solution and use 10 µl in 500 PTw for 25-30 minutes
- Wash X4
- Post fix with 70 µl 37% Formaldehide in 500 PTw
- Wash several times
- Wash in 1:1 Hyb (with pH 4.5): PTw for 10 min

Prehyb

- Remove liquid, add 1000µl Hyb buffer, incubate 10min at RT

(note: embryos can be stored in SDS-Hyb at -20C for several days)

- remove liquid, add 1000 µl Hyb buffer, place at 65C for 4 hr

Hybridization

- Dilute probe to a final concentration of 10-0.05ng/ µl (usually 1.0ng/µl) in Hyb buffer.
- Denature Hyb+probe at 80-90C max for 10 min
- Remove prehyb, add Hyb+probe

- Hyb overnight

DAY 2

- Prewarm Hyb/2XSSC/0.05XSSC to 65C
- Recover Hyb+probe (can be reused 2-3 times)

Washes

- Wash 10min in 100% Hyb at 65C
- Wash 30min in 100% Hyb at 65C
- Wash 15min in 75% Hyb/25% 2X SSC at 65C
- Wash 15min in 50% Hyb/50% 2X SSC at 65C
- Wash 15min in 25% Hyb/75% 2X SSC at 65C
- Wash 15min in 100% 2X SSC at 65C
- Wash 3X20min in 0.05X SSC at 65C X3
- Wash 10min in 75% 0.05X SSC/25% PTw at RT
- Wash 10min in 50% 0.05X SSC/50% PTw at RT
- Wash 10min in 25% 0.05X SSC/75% PTw at RT
- Wash 10min in 100% PTw at RT

Visualization of probe

- Wash 5X5min with PBT at RT X5
- Block in Boehringer-Mannheim Blocking buffer 1 hr at RT on rocker
- Incubate with Boehringer-Mannheim anti-Dig/AP (diluted in blocking buffer to **1:1500.** For instance 2 microliter in 3 ml) at 4C overnight on rocker (can also incubate 1-4 hrs at RT)

DAY 3

- Wash 4X5 min in PBT X4
- Wash 6X20 min in PBT X6
- Wash 3X10min in AP buffer X3

- Develop in AP substrate solution at RT in dark. Monitor color development (can develop slower at 4C if desired)
- Stop reaction by washing 5X5 min with PT X5
- Wash embryos 4X20min in PT X4
- Optional step (warning: do not ise during double in situ experiments): if further removal of background or intensification of signal is desired (before proceeding with this step, move embryos to microfuge tubes to prevent embryos from adhering to plastic), embryos can be dehydrated through MeOH:PT series X3 and washed 3X30 min in 100% MeOH X3 then subsequently rehydrated through PT:MeOH series X3
- Mount in Glycerol

Protocol 2

(based on Hafner et al, 2003)

FIXATION: 4% paraformaldehyde in 0.1 M PB with 0.03% Tx100 for 30 min. After initial fixation, the yolk was removed and the embryos were placed in double fixative (1% PFA and 1% glutaraldehyde in 0.1M PB, pH 7.4) for 1 hr at room temp.

Embryos were dehydrated in a methanol series and stored in 100% methanol at -20C.

DAY 1

Pretreatment

Transfer embryos to eppendorfs (1 for the antisense probe, one for the sense probe), use about 900-1000µl for each wash

- Rehydrate:

Wash 5 min in 75% MeOH/ 25% PTw

Wash 5 min in 50% MeOH/ 50% PTw

Wash 5 min in 25% MeOH/ 75% PTw

- Wash 3x5 min in PTw
- proteinase K, make a dilution of 1:10 from the stock solution and use 10 µl in 500 PTw for 15 minutes (25 µg in ml)
- Wash X3
- Post fix with 70 µl 37% Formaldehyde in 500 PTw
- Wash several times

Prehyb

- Remove liquid, add 1000 µl Hyb buffer, place at 65C for 2 hr

Hybridization

- Dilute probe to a final concentration of 70ng/ ml in Hyb buffer.
- Denature Hyb+probe at 80-90C max for 10 min
- Remove prehyb, add Hyb+probe
- Hyb overnight at 65C

DAY 2

- Prewarm Hyb 65C
- Recover Hyb+probe (can be reused 2-3 times)

Washes

- Wash 15 min in 100% Hyb at 65C X7
- Do the last wash overnight at 70C

DAY 3

- Wash 15 min in 100% Hyb at 65C X2
- Wash 15 min in 100% PT X3
- Wash 10 min in Blocking buffer X2

Visualization of probe

- Incubate with Boehringer-Mannheim anti-Dig/AP (diluted in blocking buffer plus 1% goat serum and 0.1% Tween 20. 1:1500. For instance 2 µl in 3 ml) for 2 hrs at RT
- Wash 6X20 min in PBT X6
- Wash 3X10min in AP buffer X3
- Develop in AP substrate solution at RT in dark. Monitor color development (can develop slower at 4C if desired)
- Stop reaction by washing 5X5 min with PT X3
- Wash embryos 4X20min in PT X4
- Mount in Glycerol

Protocol 3

(based on Hafner et al, 2003 and by using VIBRATOME for sectioning)

FIXATION: 4% paraformaldehyde in 0.1 M PB with 0.03% Tx100 for 30 min. After initial fixation, the yolk was removed and the embryos were placed in double fixative (1% PFA and 1% glutaraldehyde in 0.1M PB, pH 7.4) for 1 hr at room temp.

Embryos were dehydrated in a methanol series and stored in 100% methanol at -20C.

DAY 1

Pretreatment

Transfer embryos to eppendorfs (1 for the antisense probe, one for the sense probe), use about 900-1000µl for each wash

- Rehydrate:

Wash 5 min in 75% MeOH/ 25% PTw

Wash 5 min in 50% MeOH/ 50% PTw

Wash 5 min in 25% MeOH/ 75% PTw

Embryos were embedded in Agar (1gr agar + 50ml distilled water) and sectioned in slices of different thickness (50µm-80µm) by using the Vibratome. Immediately after sectioning them in PBS, we fixed the embryos in PFA for 20-30 min, then washed them in PBS.

- Wash 3x5 min in PTw
- proteinase K, make a dilution of 1:10 from the stock solution and use 10 µl in 500 PTw for 8 minutes (25 µg in ml)
- Wash X3
- Post fix with 70 µl 37% Formaldehyde in 500 PTw
- Wash several times

Prehyb

- add Hyb buffer, place at 65C for 2 hr

Hybridization

- Dilute probe to a final concentration of 70ng/ ml in Hyb buffer.

- Remove prehyb, add Hyb+probe
- Hyb overnight at 65C

DAY 2

- Prewarm Hyb 65C
- Recover Hyb+probe (can be reused 2-3 times)

Washes

- Wash 15 min in 100% Hyb at 65C X7
- Wash 15 min in 100% PT 3X
- Wash 10 min in Blocking buffer X2

Visualization of probe

- Incubate with Boehringer-Mannheim anti-Dig/AP (diluted in blocking buffer plus 1% goat serum and 0.1% Tween 20.1:1500. For instance 2 µl in 3 ml) overnight in the fridge

DAY 3

- Wash 6X20 min in PBT X6
- Wash 3X10min in AP buffer X3
- Develop in AP substrate solution at RT in dark. Monitor color development (can develop slower at 4C if desired)
- Stop reaction by washing 5X5 min with PT X3
- Wash embryos 4X20min in PT X4
- Mount in Glycerol

Protocol 4 (FISH)

(HNPP replaces the color substrates NBT/BCIP, so the detection protocol is more or less the same. Solutions are slightly different and as well as the concentration of the active compounds).

FIXATION: 4% paraformaldehyde in 0.1 M PB with 0.03% Tx100 for 30 min. After initial fixation, the yolk was removed and the embryos were placed in double fixative (1% PFA and 1% glutaraldehyde in 0.1M PB, pH 7.4) for 1 hr at room temp.
Embryos were dehydrated in a methanol series and stored in 100% methanol at -20C.

DAY 1

Pretreatment

Transfer embryos to eppendorfs (1 for the antisense probe, one for the sense probe), use about 900-1000µl for each wash

- Rehydrate:

Wash 5 min in 75% MeOH/ 25% PTw
Wash 5 min in 50% MeOH/ 50% PTw
Wash 5 min in 25% MeOH/ 75% PTw

- Wash 3x5 min in PTw
- proteinase K, make a dilution of 1:10 from the stock solution and use 10 µl in 500 PTw for 15 minutes (25 µg in ml)
- Wash X3
- Post fix with 70 µl 37% Formaldehyde in 500 PTw
- Wash several times

Prehyb

- Remove liquid, add 1000 µl Hyb buffer, place at 65C for 2 hr

Hybridization

- Dilute probe to a final concentration of 70ng/ ml in Hyb buffer.
- Denature Hyb+probe at 80-90C max for 10 min

- Remove prehyb, add Hyb+probe
- Hyb overnight at 65C

DAY 2

- Prewarm Hyb 65C
- Recover Hyb+probe (can be reused 2-3 times)

Washes

Wash 15 min in 100% Hyb at 65C X7

Do the last wash overnight at 70C

DAY 3

- Wash 15 min in 100% Hyb at 65C X2
- Wash 15 min in 100% PT X3
- Wash 10 min in Blocking buffer X2

Visualization of the probe

(from Roche product description sheet, available on the Roche website)

Required reagents and solutions

Buffer 1:

100 mM Tris-HCl, 150 mM NaCl, pH 7.5 (20° C)

Buffer 2: Blocking solution

0.5% Blocking reagent in buffer 1

Buffer 3: Washing buffer

0.05% Tween > 20 in buffer 1

Buffer 4: Detection buffer

100 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl2, pH 8.0 (20° C)

Fast Red TR solution

25 mg/ml Fast Red TR in redist. H2O. Store at 2-8° C in the dark. Do not use solution longer than 4 weeks as progressive precipitation occurs.

Immunological detection

1. Dilute anti-digoxigenin-AP conjugate 1:500 in buffer 2 (blocking solution). It is stable for only 12 h at 2-8° C.
2. Apply 20–100µl of diluted anti-digoxigenin-AP to the slide, cover with coverslip.
3. Incubate for 1 h at 37° C in a moist chamber.
4. Wash the slide three times with buffer 3 (washing buffer) for 10 min each at 15-25° C.
5. Wash the slide twice with buffer 4 (detection buffer) for 10 min at 15-25° C.

Preparation of HNPP/Fast Red TR mix

Mix the following components in a sterile tube:
10µl HNPP (10 mg/ml in dimethylformamide)
10µl Fast Red TR solution.
Note: Do not use the solution longer than 4 weeks as progressive precipitation occurs.
1 ml buffer 4 (detection buffer)
Pass the HNPP/Fast Red TR mix through a 0.2µm nylon syringe filter before use.
(It is recommended to use the HNPP/Fast Red TR mix freshly prepared. It may be stored up to a few days at 2-8° C protected from light.)

Staining

1. Apply 20–100µl of the HNPP/Fast Red TR mix to the slide, cover with a coverslip, and incubate for 30 min at 15-25° C.
2. Wash the slide with redist. H_2O for 10 min.

Counterstaining

The use of DAPI for counterstaining of HNPP-detected probes is recommended.
1. Incubate slides for 5 min in the dark at 15-25° C in 50 ml PBS containing 1µl DAPI solution (5 mg/ml).
2. Wash 2–3 min under running water.
3. Allow to air-dry in the dark.

Mounting

Mount in Glycerol

Fluorescent detection

The signals are detected by fluorescence microscopy. Since HNP/Fast Red TR emits fluorescence in a wide range between 540–590 nm with a maximum at 562 nm, filter sets for fluorescein or rhodamine can be used.

SUMMARY

In the current project, the embryonic and postembryonic development of the brain of the parthenogenetic Marbled crayfish (*Procambarus* sp.) (Malacostraca, Decapoda, Astacida) was investigated. The Marbled crayfish is characterized by a genetically uniform progeny, high fertility, high reproduction rate and a published staging system of its embryonic development is available. Its general development is more or less identical to that of other crayfish and for this reason it is considered a suitable model organism for developmental studies.

The main aim of the project was to study the formation of the main brain scaffold and the mechanisms underlying this process. The brain forms during embryogenesis from an initial thin layer of cells (neuroectoderm) that unfolds into a three dimensional complex structure. This process involves both an intense mitotic activity of progenitor cells (neuroblasts) and a series of morphogenetic movements that lead to a mature and functioning structure of the brain. Analyses were based on immunohistochemical techniques, use of proliferation markers (e.g. 5-Bromo-2-deoxyuridine and 5-ethynyl-2'-deoxyuridine) and confocal laser-scan microscopy. Moreover, the use of a specific monoclonal antibody against the protein expressed by *engrailed* (a segment polarity gene) identified single neuronal cells that were compared with data available for Insecta. In this way, both information on the neurobiology of the Marbled crayfish and on its phylogeny were acquired.

By applying molecular techniques, three genes involved in neuronal development (*Pax6*, *atonal* and *orthodenticle*) were isolated and characterized. Analysis on the primary sequence showed a high level of evolutionary conservation of the genes within Arthropoda. These data set the base for future analysis on the embryonic expression of these genes once an appropriate protocol for *in situ* hybridization will be available.

One important feature of the crayfish brain is the high plasticity that it shows not only during embryogenesis but throughout its life span. The brain in fact does not stop to

develop and after embryogenesis the central olfactory pathway receives newly born olfactory local interneurons and projection neurons that replace old neurons or simply are added to the previous neuronal population. These neurons are provided by a specific post-embryonic structure, the neurogenic niche, that emerges at the end of embryogenesis. By monitoring the activity of NBs by using proliferation markers and performing pulse-chase experiments, it was possible to discover when and how the niche starts forming and functioning. The niche arises from a group of embryonic neuroblasts that proliferate and initially gives rise to the Deutocerebral Proliferative System which in turn divides into a storage niche and two proliferative zones (LPZ and MPZ), each of them characterized by unique features such as types of cells and structure. While LPZ and MPZ contain mitotically active cells that contribute to the adult neurogenic system since the beginning of the post-embryonic life, the storage niche starts generating progeny later in juveniles.

The hypothesis of a storage niche instead of an active niche (as LPZ and MPZ are) could explain the replacement of stem cells in the brain during the life span of the crayfish and it could explain why such apparatus (three “niches” linked together) is built to face the adult life. Replacement of stem cells is in fact a phenomenon which has not been solved yet by the models currently available (Sullivan et al., 2007a and Song et al., 2009).
The identity of stem cells hosted within the niche and the two proliferative zones is unknown. In the current project, once again, my model suggests two different types of cells, stem cells and progenitor cells. Stem cells reside within the storage niche and they send off progenitor cells along the pathway which eventually become resident stem cells within the proliferative zones. This model explains the continuous activity within the PZs, made evident by the pulse chase experiments. Surely, future studies should address these questions in order to understand the potentiality of these cells and their main characteristics. The topic of adult neurogenesis is in fact a fascinating field of research which involves stem cells biology and regulatory mechanisms that are just now started to be explored and understood. This challenging field of research holds many future perspectives, because some of the main characteristics of the adult neurogenesis in crustaceans are shared with vertebrates, in particular with mammals.

ZUSAMMENFASSUNG

Die vorliegende Dissertation widmet sich der Analyse von embryonalen und postembryonalen Entwicklungsprozessen im Gehirns des Marmorkrebs‘, eines pathenogenetischen Flusskrebses (*Procambarus* sp.; Malacostraca, Decapoda, Astacida). Der Marmorkrebs bringt eine genetisch einheitliche Nachkommenschaft hervor. Die Tiere zeichnen sich durch eine hohe Reproduktionsrate aus, und für die Embryonen liegt ein publiziertes Stadiensystem für die Ontogenese vor. Dabei ist der Entwicklungsgang des Marmorkrebs‘ dem anderer Flusskrebse sehr ähnlich. Zusammengenommen kann der Marmorkrebs als ein geeignetes und repräsentatives Modellsystem für entwicklungsbiologische Studien innerhalb der Decapoda gelten.

Ziel der Studie war, die Entfaltung des Gehirnes zu beschreiben und mögliche Entwicklungsmechanismen zu beleuchten. Am Anfang der Embryogenese wird das Gehirn als dünne neuroectodermale Zellage zu Grunde gelegt, aus der sich später die dreidimensionale, komplexe Struktur entwickelt. Dieser Prozess wird initiiert durch eine intensive mitotische Aktivität von neuronalen Vorläuferzellen (Neuroblasten) und ist begleitet von komplexen morphogenetischen Bewegungen die schließlich die funktionale Struktur des reifen Gehirns hervorbringen. Methodisch kamen in der Studie immunhistochemische Techniken sowie Mitosemarker zum Einsatz (z. B. 5-Bromo-2-deoxyuridine und 5-ethynyl-2’-deoxyuridine), die Auswertung erfolgte vorwiegend mit confocaler Laser-Scan Mikroskopie. Des Weiteren wurde ein monoklonaler Antikörper gegen das vom *engrailed* Gen exprimierte Protein (ein Segmentpolaritätsgen) genutzt, um einzelne Neurone individuell zu identifizieren und deren Position und Anzahl mit der ähnlicher Neurone im embryonalen Insektengehirn zu vergleichen. Mit diesen Ansätzen konnten Erkenntnisse sowohl zu neurogenetischen Mechanismen als auch zu evolutiven Aspekten der Neurogenese gewonnen werden.

Mit Hilfe von molekularen Techniken wurden Orthologe von drei Genen charakterisiert, die in anderen Arthropoden erwiesenermaßen eine wichtige Rolle in der Neurogenese spielen: *Pax6*, *atonal* und *orthodenticle.* Analysen der Primärsequenz belegten, dass diese

Gene innerhalb der Arthropoda hoch konserviert sind. Sobald ein funktionsfähiges Protokoll für die *in situ* Hybridisierung etabliert ist, können diese Daten als Basis dienen für zukünftige Analysen, welche die Rolle, die diese Gene in der Neurogenese von Decapoda spielen, beleuchten.

Es ist inzwischen gut belegt, das sich das Gehirn decapoder Crustacea während der gesamten Lebensspanne der Tiere durch einen hohen Grad neuronaler Plastizität auszeichnet. So werden auch nach der Embryogenese im zentralen olfaktorischen Pfad neue olfaktorische Interneurone und Projektionsneurone angelegt, die alte Nervenzellen ersetzen und die bestehende neuronale Population vermehren. Diese neuen Neurone werden durch die mitotische Aktivität einer spezifischen Struktur hervorgebracht, der neurogenetischen Nische, die sich gegen Ende der Embryogenese aus einer Gruppe von Neuroblasten entwickelt. Die neurogenetische Nische ist gemeinsam mit zwei weiteren Proliferationszonen Teil des "Deutocerebral Proliferative System" (DPS) im adulten Gehirn der Decapoda. In der vorliegenden Arbeit wurde mit Hilfe von Mitosemarkern der embryonale Ursprung des "Deutocerebral Proliferative System" sowie dessen neurogenetische Aktivität in der Postembryonalphase untersucht.

Sowohl die neurogenetische Nische als auch die beiden Proliferationszonen (LPZ und MPZ) zeichnen sich durch individuelle Charakteristika der dort vorkommenden Zelltypen aus. Während die laterale Proliferationszone (LPZ) mitotisch aktive Zellen enthält, die bereits direkt im Anschluss an die Embryogenese Nachkommenschaft zum DPS beiträgt, wird die neurogenetische Nische erst später, nämlich in den juvenilen Tieren mitotisch aktiv. Sie fungiert anfangs also eher als Struktur, die neuronale Stammzellen, die momentan mitotisch in aktiv sind, in einem besonderen Mikromillieu bewahrt, bis sie gebraucht werden. Aus diesem Vorrat an Zellen werden dann, möglicherweise auf bestimmte, noch nicht untersuchte zelluläre Signale hin, selektiv einige Zellen wieder aktiv, teilen sich, und bringen Nachkommenschaft in die Proliferationszonen ein. Aus diesem Zusammenspiele einer „Speichernische“ mit zwei aktiven Proliferationszonen ließe sich erklären, wie lebenslang Stammzellen zur Neurogenese zur Verfügung stehen können, eine Frage die momentan intensiv diskutiert wird (Sullivan et al., 2007a and

Song et al., 2009). Die genaue Identität der Stammzellen in der „Speichernische“ und den Proliferationszonen konnte auch in dieser Arbeit nicht abschließend aufgeklärt werden. Aus den vorliegenden Daten lässt sich aber die Hypothese ableiten, dass in der „Speichernische“ Stammzellen vorliegen, die Vorläuferzellen hervorbringen, die entlang sich eines migratorischen Stromes in die Proliferationszonen bewegen, um dort wiederum Stammzelleigenschaften anzunehmen. Aus diesem Modell lässt sich die dauerhafte mitotische Aktivität in den Proliferationszonen erklären, wie sie durch „Pulse-Chase“ Experimente mit den Mitosemarker belegt wurde. Weitere Versuchsreihen sind nötig, um diese Hypothese zu erhärten. Das untersuchte System birgt ein großes Potential für zukünftige Untersuchungen auf dem Feld der Stammzellbiologie und der Untersuchung von regulatorischen Mechanismen, die die adulte Neurogenese steuern, auch mit Hinblick auf ähnliche, intensiv untersuchte Prozesse im Vertebratengehirn wie der adulten Neurogenese im Hippocampus oder dem olfaktorischen System.

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Thanks to all of you

Silvia Sintoni

DECLARATION

I confirm that the work presented in this dissertation is my own. Where information has been cited from other sources, I confirm that this has been indicated in the dissertation.

Ulm
Silvia Sintoni
Signature:

CURRICULUM VITAE



PERSONAL DATA

Name and Surname: Silvia Sintoni
Date and Place of birth: 08 November 1975, Forlì, Italy
Status: not married. One kid
Residence: Via Mario Quartaroli 1, 47100 Forlì, Italy
Current position: PhD candidate
Mobile number: 0039-347-8821418
Email address: silviasintoni@hotmail.com
MPI email address: ssintoni@ice.mpg.de

ACADEMIC BACKGROUND

- 09 March 2000 Diploma di Laurea in Biological Sciences, University of Bologna, Italy. 110/110 *cum laude*.
Thesis: VARIATION OF METABOLITES DURING THE FUNCTIONAL ANAEROBIOSIS IN *CARCINUS MEDITERRANEUS* (CZERNIAVSKY)
Supervisors: Prof. Otello Cattani, Prof. Paolo Cortesi (Department of Biochemistry)

- February 2004-June 2005 Master of Applied Science in Marine Biology, James Cook University, Townsville, North Queensland, Australia.
Thesis: INTERACTION BETWEEN CORAL CALMODULIN (CaM) AND GREEN FLUORESCENT PROTEIN (GFP) BY USING THE TWO-HYBRID SYSTEM
Supervisors: Prof. David Miller, Prof. Bette Willis (Department of Molecular Biology)

- June 2006-February 2010 PhD candidate, University of Ulm, Department of Neurobiology.
Marie Curie Fellowship, MOLMORPH network (www.molmorph.net)
Thesis: NEUROGENESIS IN THE BRAIN OF THE MARBLED CRAYFISH
Supervisors: Prof. Dr. Steffen Harzsch (University of Greyswald), Prof. Dr. Harald Wolf (University of Ulm).

ACADEMIC ACTIVITIES

POSTERS

- 29th March-1st April 2006: 7th Goettingen Meeting of the German Neuroscience Society, Goettingen, Germany Society.

Poster: Analysing cell proliferation by using a mitotic marker (BrdU) during neurogenesis in crayfish
Authors: Silvia Sintoni, Kathia Fabritius Vilpoux, Steffen Harzsch

- 22nd-27th July 2007: 8th International Congress of Neuroethology at the University of British Columbia, Vancouver, Canada.

Poster: Brain development in Crustacea: expression of the segment polarity gene *engrailed*
Authors: Silvia Sintoni, Kathia Fabritius Vilpoux, Steffen Harzsch

- 27th September 2007. 6th Biannual Symposium of the International Max Planck Research School (IMPRS).

Poster: Development of the Neurogenic Niche in olfactory lobes of Marbled crayfish embryos: the beginnings of adult neurogenesis
Authors: Silvia Sintoni and Steffen Harzsch

TALKS

- "The development of the niche: comparison between crayfish and lobster" 17th July 2007, Wellesley College, Wellesley, Massachusset, United States

- "Brain development in Crustacea" 27–30th November 2007, **Molmorph-Zoonet** Barcelona, Spain

- "Neurogenesis during and after embryogenesis in the Marbled crayfish" 16–20th September 2008: **Molmorph** meeting, Vienna, Austria

COURSES IN:

ENGLISH: Ireland, TOEFL test, 2001.
PHOTOSHOP: University of Ulm, Germany, October 2006
CONFOCAL MICROSCOPY: Carl Zeiss, Jena, Germany, 2007
IMMUNOHISTOCHEMISTRY: University of Copenaghen, Denmark, 2006
EVO-DEVO: Venezia, Prof. Alessandro Minelli.

TRAININGS:

- 4th June- 20th July 2007: Research work at the Wellesley College, Wellesley, Massachusset, United States, in collaboration with Prof. Barbara Beltz. Project: adult neurogenesis in Crustacea (American lobster and crayfish, genus *Procambarus*). Development of the neurogenic niche responsible for the production of new cells in the deutocerebrum of the adult brain.

- 13 October– 30 November 2007: training in molecular techniques at Prof. Dr. Pedro Martinez's lab in Barcelona, Spain. *In situ* hybridization and cloning techniques were applied to *Procambarus* sp. in order to investigate the expression pattern of specific genes involved in brain development during embryogenesis.

- 17-29 February 2008: training in molecular techniques at Niko Prpic's lab in Goettingen, Germany, in order to improve skills and methods of *in situ* and focus efforts and experiments on embryos of different stages of *Procambarus* sp.

PUBLICATIONS

1. Sintoni S, Fabritius-Vilpoux K, Harzsch S (2007) The Engrailed-expressing secondary head spots in the embryonic crayfish brain: examples for a group of homologous neurons in Crustacea and Hexapoda? Development Genes and Evolution 217, 791-799

2. Harzsch S,. Müller C H G, Rieger V, Perez Y, **Sintoni S**, Sardet C, Hansson B (2009) Fine structure of the ventral nerve centre and interspecific identification of individual neurons in the enigmatic Chaetognatha. Zoomorphology. 53-73

WORKING ACTIVITIES

- 2000-2001: Italian teacher in an international language school, Lymerick County, Ireland.
- 2001-2004: marine biologist and tour guide on safari boats in Maldives, Indian Ocean. Dive Master License.
- October-December 2005: laboratory technician at the University of Algarve, Portugal.
- January-May 2006: biologist for schools. Atlantide Cooperative, Cervia, Italy. Environmental activities for students and adults.

HOBBIES

Diving, traveling