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Molecular and cellular characterisation of neurogenesis control and addiction behaviour in zebrafish

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Abstract

Neurogenesis control is crucial for the correct development of the nervous system from early embryonic stages to the fully developed adult organism. Premature differentiation not only prevents the formation of later-born cell types – it also causes disorganization of the shape and cytoarchitecture of the brain. In addition, progenitors that are maintained life-long provide sources for brain plasticity and regeneration and there is increasing evidence that neurogenesis plays a role in mood and behaviour in the fully developed adult organism. This project uses the advantages of zebrafish as a model for embryonic development, but also as a model for use in behavioural studies. The main aim of my PhD work is to add to the understanding of the molecular and cellular processes that maintain neural progenitors within the vertebrate central nervous system (CNS) and also to set the basis for understanding the impact of neurogenesis on brain physiological processes such as reward and drug reinforcement. To this end I isolated and characterised a novel member of the zebrafish Hairy/Enhancer of Split (hairy/E(spl)) family – *her8a*. In addition, I also investigated the molecular mechanisms of reward and drug reinforcement, through the characterisation of a mutant that fails to respond to amphetamine, *no addiction* (*nad*). Analysis of the mutant reinforces the link between behaviour and neurogenesis.

The neural plate of the early embryo is divided into areas of neurogenesis ('proneural clusters') and areas where neurogenesis is actively inhibited ('progenitor pools'). In the proneural clusters neurogenesis promoting genes, such as *neurog1,* are expressed in a salt and pepper pattern with members of the Hairy/E(spl) factors, such as *her4* (Takke et al., 1999). In these proneural clusters neurogenesis is controlled through the process of lateral inhibition. Outside the proneural clusters, neurogenesis is actively inhibited in the progenitor pools, which are characterised by the expression of certain *hairy/E(spl)* genes, such as *her3*, *her5*, *her9* and *her11*/*him*. For example, the midbrain-hindbrain domain of the vertebrate embryonic neural plate displays neuronal differentiation organised around a neuron-free zone at the midbrain-hindbrain boundary (mhb). Forced neurogenesis in this area prevents the continued expression of genes defining mhb identity. The morpholino-mediated knockdown of *her5* causes ectopic neurogenesis, and thus loss of the medial mhb progenitor zone. Basic Helix Loop Helix (bHLH) transcription factor family members, such as Her5, form hetero- or homo-dimers in order to carry out their functions as repressors or activators. The elucidation of which bHLH factors form dimers with Her5 would provide an insight as to the mechanisms of the Her5 neurogenesis inhibition. To this end a yeast-2-hybrid experiment was carried out in order to identify factors that bind to Her5. I cloned and characterised the expression of the most promising candidates from the screen. One factor, Her8a, is particularly promising, as it is expressed at the mhb from before the start of segmentation. Its expression, while broad in the early embryo, becomes increasingly restricted and it is only expressed in proliferation zones in the adult brain. Experiments using morpholino mediated knockdown of *her8a* and the overexpression of *her8a* establish Her8a as a novel negative regulator of neurogenesis in the embryonic midbrain-hindbrain domain and a manuscript describing this work is currently in preparation. *her8a*'s sensitivity to Notch changes throughout development – at early stages *her8a* does not require Notch for its expression, however it is sensitive to Notch signalling. At later stages *her8a* requires Notch for its expression. This indicates that the ability of *her* genes to respond to Notch is not fixed, but they respond according to their cellular context.

Abstract

no addiction (*nad*) is a dominant mutation that was isolated in a screen for its failure to show conditioned place preference response to amphetamine in our laboratory. My task was to characterise this mutant at the molecular level in order to contribute to the understanding of the mechanisms leading to reward and drug addiction. This work has been compiled in an article, currently under revision at Genome Biology. To this end I devised a series of microarray experiments that were then combined to specifically isolate genes implicated in both the non-response to amphetamine in the wildtype as well as the failure of the mutant to respond to amphetamine – referred to in this work as the "reward pool". I analysed this pool using Gene Ontology (GO) enrichment analysis and network analysis. Network analysis linking proteins according to function is based on experimentally derived protein-protein interactions through literature curation. As there are comparatively less abstracts on zebrafish than on mammalian subjects, commercial network analysis software does not yet provide a large number of links. Therefore, in collaboration with the Institute for Bioinformatics and Systems Biology, I participated in the development of a database - zfishDB (http://mips.gsf.de/zfishdb/) - that uses zebrafish information as well as information derived from the mammalian homologues of zebrafish genes. The bioinformatics analysis of the microarray results implicates for the first time the reuse of developmental transcription factors in reward and drug reinforcement events. In addition, I used the bioinformatic analysis to choose a subset of genes for validation using qPCR and in situ hybridisation. In situ hybridisation revealed that a subset of these genes is down-regulated in neurogenic zones upon amphetamine administration. This lead to a further project, with investigated the influence of amphetamine on proliferation and differentiation in the adult brain. Here I was able to show that amphetamine leads to premature differentiation of adult progenitors.

In summary, this thesis contributes to a greater understanding of neurogenesis inhibition and to the molecular cascades involved in reward/drug reinforcement. It provides the basis for further studies looking at the mechanisms of the function of the chosen candidate genes and studies looking at the effects of amphetamine on neurogenesis in the adult.

Zusammenfassung

Die Steuerung der Neurogenese ist kritisch für die richtige Entwicklung des Nervensystems von den frühen embryonalen Stadien zum voll entwickelten ausgewachsenen Organismus. Verfrühte Differenzierung kann nicht nur die Ausformung später entstehender Zelltypen verhindern- es verursacht auch die Disorganisation der Form und Zellarchitektur des ganzen Gehirns. Zusätzlich stehen die Ursprungszellbestände lebenslang als Quellen für Gehirnplastizität und Regeneration zur Verfügung und es gibt zunehmend mehr Hinweise darauf, dass die Neurogenese eine Rolle bei Stimmung und Verhalten ausgewachsener Organismen spielt. Das Hauptziel meiner Doktorarbeit ist ein Beitrag zum Verständnis darüber, wie Ursprungszellbestände spezifiziert und aufrechterhalten werden. Zu diesem Zweck habe ich ein neues Mitglied der Zebrafisch "Hairy/Enhancer of Split" Familie - Her8a- isoliert und charakterisiert. Zusätzlich habe ich die molekularen Mechanismen von Belohnung und Drogenbestärkung untersucht, durch Charakterisierung einer nicht auf Amphetamin reagierenden Mutante - *no addiction* (*nad*).

Die neural plate des frühen Embryos ist aufgeteilt in Gebiete mit Neurogenese ("proneurale Clusters") und Gebiete, in denen Neurogenese aktiv unterdrückt wird ("Vorläuferzellpopulationen"). In den proneuralen Clusters werden Neurogenese befördernde Gene wie *ngn1* in einem gesprenkelten Muster mit Mitgliedern der *hairy/E(spl)* Genen wie *her4* exprimiert. In diesen proneuralen Gruppierungen wird Neurogenese durch den Prozess lateraler Unterdrückung gesteuert. Außerhalb der proneuralen Gruppierungen wird die Neurogenese aktiv in den Vorläuferreservoirs unterdrückt, die durch die Expression bestimmter *hairy/E(spl)* Genen wie *her3*, *her5*, *her9* und *her11/him* charakterisiert werden. Die Mittelhirn-Hinterhirn Domäne der embryonalen Ebene der Wirbeltiere zeigt eine neuronale Differenzierung, die um eine neuronenfreie Zone an der Mittelhirn-Hinterhirn Grenze (mhb) herum organisiert ist. Erzwungene Neurogenese in diesem Gebiet verhindert die fortgesetzte Expression von Genen, die die mhb-Identität definieren. Ein Morpholino-vermitteltes Knockdown von *her5* verursacht ektopische Neurogenese und dadurch den Verlust der mittleren mhb Vorläuferzellpopulationen. Mitglieder der basic Helix-Loop-Helix (bHLH) Familie wie Her5 formen hetero- oder homo-Dimere aus um ihre Funktion als Unterdrücker oder Aktivator auszuführen. Die Aufklärung welche bHLH Faktoren Dimere mit Her5 formen trägt zu einem tieferen Verständnis der Mechanismen der Neurogeneseunterdrueckung bei. Zu diesem Zweck wurde ein Yeast-2-Hybrid Experiment durchgeführt um Faktoren zu identifizieren, die an Her5 binden. Ich habe die vielversprechendsten Kandidaten des Screenings kloniert und die Expression charakterisiert. Der Faktor Her8a ist besonders interessant, da er im mhb noch vor dem Segmentierungsbeginn exprimiert wird. Die breite Expression von Her8a im frühen Embryo wird zunehmend eingeschränkt und wird im ausgewachsenen Hirn nur in den Proliferationsgebieten exprimiert. Experimente mit Morpholino-vermitteltem Knockdown von her8a und der Überexpression von *her8a* begründen Her8a als negativen Regulator der Neurogenese. Die Empfindlichkeit von Her8a auf Notch ändert sich im Laufe der Entwicklung - in frühen Stadien benötigt *her8a* kein Notch für seine Expression, es reagiert jedoch auf Notchsignalisierung. In späteren Stadien benötigt *her8a* Notch für seine Expression. Dies weist darauf hin, dass die Fähigkeit der *her* Gene auf Notch zu reagieren nicht statisch ist, sondern dass ihre Reaktion vom zellularen Kontext abhängt.

no addiction (*nad*) ist eine dominante Mutation, die in unserem Labor bei einem Screening auf das Versagen erlernter Aufenthaltsortspräferenz unter Einfluss von Amphetamin isoliert wurde. Meine Aufgabe war die Charakterisierung dieser Mutante auf molekularer Ebene, um zum Verständnis der zu Belohnung und Drogensucht führenden Mechanismen beizutragen.

Zu diesem Zweck entwickelte ich eine Serie von Microarray Experimenten, die danach kombiniert wurden um spezifisch Gene zu isolieren, die sowohl für die Unempfindlichkeit auf Amphetamin im Wildtyp als auch für das Versagen der Mutante auf Amphetamin zu reagieren verantwortlich sind- in dieser Arbeit genannt das ‚reward pool'. Ich untersuchte dieses ‚reward pool' mittels Ontology (GO) Anreicherungsanalyse und Netzwerkanalyse. Netzwerkanalyse, die Proteine nach ihrer Funktion verknüpft, ist basiert auf experimentell abgeleiteten Protein-Protein Interaktionen aus Literaturrecherche. Da vergleichsweise weniger Abstrakts in Bezug auf Zebrafisch zur Verfügung stehen als für andere Säugetiere, liefert kommerzielle Netzwerkanalysesoftware derzeit noch keine große Anzahl an Verbindungen. Aus diesem Grund habe ich in Zusammenarbeit mit dem Institute for Bioinformatics and Systems Biology an der Entwicklung einer Datenbank -zfishDB (http://mips.gsf.de/zfishdb/)- mitgearbeitet, die sowohl Zebrafischdaten als auch Daten von Säugetierhomologen nutzt. Diese Bioinformatikarbeit impliziert zum ersten Mal die Wiederverwendung der Entwicklungstranskriptionsfaktoren in den durch Drogen und Belohnung ausgelösten Vorgängen. Zusätzlich habe ich die Bioinformatikanalyse dazu verwendet eine Untermenge von Genen fuer die Valiedierung mittels qPCR und in-situ Hybridisierung auszuwählen. In situ Hybridisierung zeigte, dass eine Untermenge dieser Gene in Neurogenesegebieten nach Amphetamingabe heruntergeregelt wird. Dies führte zu einem weiteren Projekt, das den Einfluss von Amphetamin auf Verbreitung und Differenzierung im ausgewachsenen Gehirn untersucht. An dieser Stelle konnte ich zeigen, dass Amphetamin zu einer verfrühten Ausdifferenzierung erwachsener Vorläuferzellen führt.

Zusammenfassend trägt die vorliegende Arbeit zu einem tieferen Verständnis der Neurogeneseinhibition und der molekularen Kaskaden im Zusammenhang mit Belohnung und Drogenbestärkung bei. Eine Basis wurde geschaffen fuer weitere Studien der Mechanismen und Funktionen der ausgewählten Genkandidaten und für Studien der Neurogenese in ausgewachsenen Individuen.

Index

1. Introduction

The main aim of my PhD work is to contribute to the understanding of the molecular and cellular processes that maintain neural progenitors within the vertebrate central nervous system (CNS) and also to set the basis for understanding the impact of neurogenesis on brain physiological processes such as reward and drug reinforcement. To this aim I used the advantages of the zebrafish as a model for both embryonic studies and also adult behaviour.

My introduction is comprised of three major parts – 1) neurogenesis control in the vertebrate embryo, 2) mechanisms of addiction and 3) adult neurogenesis. In the first part, I will begin by out-lining the formation and patterning of the early zebrafish nervous system, with a focus on the mechanisms allowing the formation of 'proneural clusters' (areas of neurogenesis) and 'progenitor pools' (areas of active inhibition of neurogenesis). Members of the basic Helix-Loop-Helix (bHLH) superfamily of transcription factors, such as Her factors, play crucial roles in this process, and indeed a focus of this work is on Her5 and its potential binding partner Her8a. Therefore, I will end this first section with review of the phylogeny, function and functional mechanisms of bHLH factors.

In a second section I will discuss how addiction to drugs of abuse forms and how we can study addiction processes and reward in the laboratory. I will also focus here on zebrafish as a model organism for behaviour and behavioural disorders. Lastly I will discuss adult neurogenesis, with particular focus on the effects of drugs of abuse on adult neurogenesis.

1.1 Neurogenesis in the embryo

1.1.1 Formation of the nervous system

At the most basic level, the function of the nervous system is controlled by individual cells – the neurons. In order to properly create the diversity and connectivity of the fully-developed nervous system, each neuron must be directed to differentiate at a particular time and position and to adopt a particular fate. Neurogenesis is a multistep process that begins with neural induction and ends with the differentiation of functional neurons (Appel and Chitnis, 2002). The process of neurogenesis involves several successive steps, characterized by specific signalling events (Wilson and Edlund, 2001) and by the expression of different sets of transcription factors (Bally-Cuif and Hammerschmidt, 2003; Bertrand et al., 2002). First the neural plate is formed, in the process of neural induction. Secondly, during the commitment phase, areas of neurogenesis ('proneural clusters') are defined within the neural plate by the action of inhibitors and activators of neurogenesis. Finally, neural progenitors differentiate into neurons or glia (see figure 1). A timeline of these steps is depicted, along with the embryonic stages especially relevant to this thesis, in figure 2. I will discuss these processes in detail in the following pages.

1.1 Neurogenesis in the embryo

The zebrafish egg and early zebrafish embryo display to a large extent axial symmetry until the onset of gastrulation ('shield' stage). During gastrulation the cells of the future neural plate converge towards the midline and concomitantly expand along the anterior-posterior axis, driven by convergence-extension movements (Solnica-Krezel and Cooper, 2002) and active migration (Varga et al., 1999). After gastrulation, during the early somitogenesis stages, the first morphologically visible processes of neurulation take place. First, two lateral thickenings become visible. Then, from the 6 to 10 somite stages the neural plate condenses and the two lateral thickenings move towards the midline in an infolding movement, resulting in the formation of the 'neural keel'. The lateral cells end up more dorsal in the keel, while the cells that were medial go to deeper ventral positions (Papan and Campos-Ortega, 1994). The keel becomes round, and forms a cylindrical structure called the 'neural rod' that detaches from the adjacent skin ectoderm (see also figure 3).

Neural induction is the first step in the neurogenesis cascade that specifies the neural plate (Stern, 2005). In this process the embryonic ectodermal cells are specified towards the more restricted fate of neuroectodermal cells, that is neural stem or precursor cells. Several secreted signalling components are responsible for this transition. Experiments in frog have shown that ectodermal cells have an innate tendency to differentiate into neural tissue if they are not inhibited by bone morphogenetic proteins (BMPs). The BMP inhibitors Noggin, Chordin and Follistatin allow the adoption of non-neural fates. These factors are secreted from cells of the primary organiser during gastrulation (Wilson and Edlund, 2001).

Other factors, such as Fibroblast growth factors (Fgfs) and Insulin-like growth factors (Igfs) act as neural inducing signals before the onset of gastrulation, and therefore before BMP inhibition by the primary organiser. These signals converge with Smad1. Phosphorylation of Smad1 through BMP signalling is activating, while phosphorylation in the Smad1 linker region through Fgf/Igf signalling is repressive (Kretzschmar et al., 1997; Pera et al., 2003).

All of these processes that modulate neural induction led to a broad upregulation of the neural plate markers *sox2/3* (Chapouton and Bally-Cuif, 2004; Stern, 2005). By the end of gastrulation the responsiveness to neural induction has ceased and the cells that have been induced are now either committed to neural differentiation or to the neural progenitor state (Wilson and Edlund, 2001).

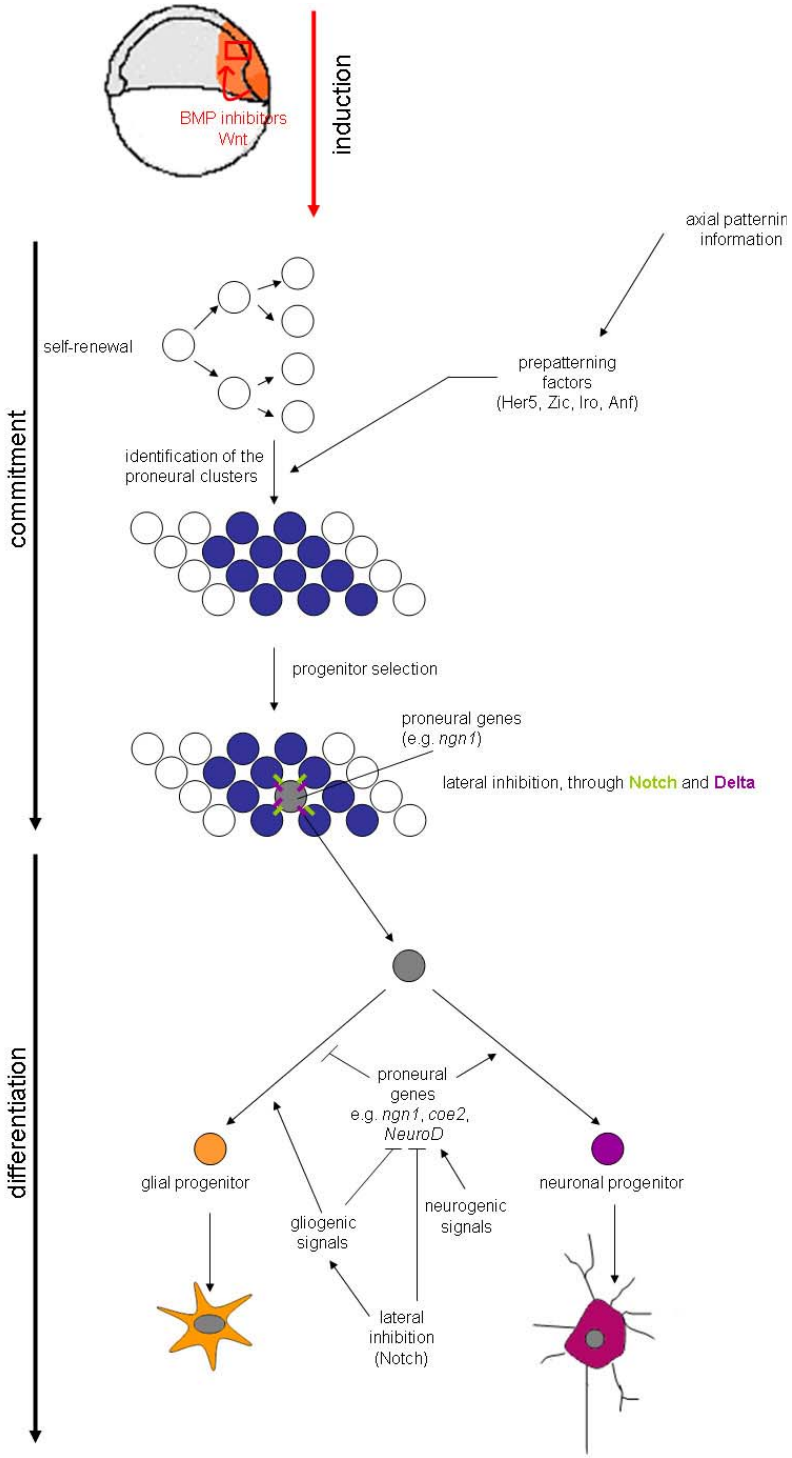

Figure 1. Neurogenesis in vertebrates takes place over several successive steps and involves specific signalling events. 1. During gastrulation the neural plate is identified through the process of neural induction through the interaction between three signalling pathways (Fgf, Wnt and Bmp). Fgf signalling represses Bmp and activates an independent pathway necessary for the progression towards the neural fate. Fgf signalling is itself modulated by Wnt from the embryonic margin. 2. In the process of neural commitment proneural clusters are defined within the neural plate. A restricted number of neural progenitors from each cluster is further selected by the process of lateral inhibition (see '1.1.2 The delimitation of proneural fields by prepatterning factors', section: Lateral inhibition selects neural progenitors within the proneural clusters.), in which the expression level of proneural genes in the selected progenitors is elevated, resulting in the final commitment to the neural lineage. Committed progenitors can give rise to both neural and glial cells. 3. Committed progenitors generally first generate neurons and then glial cells in the process of differentiation.

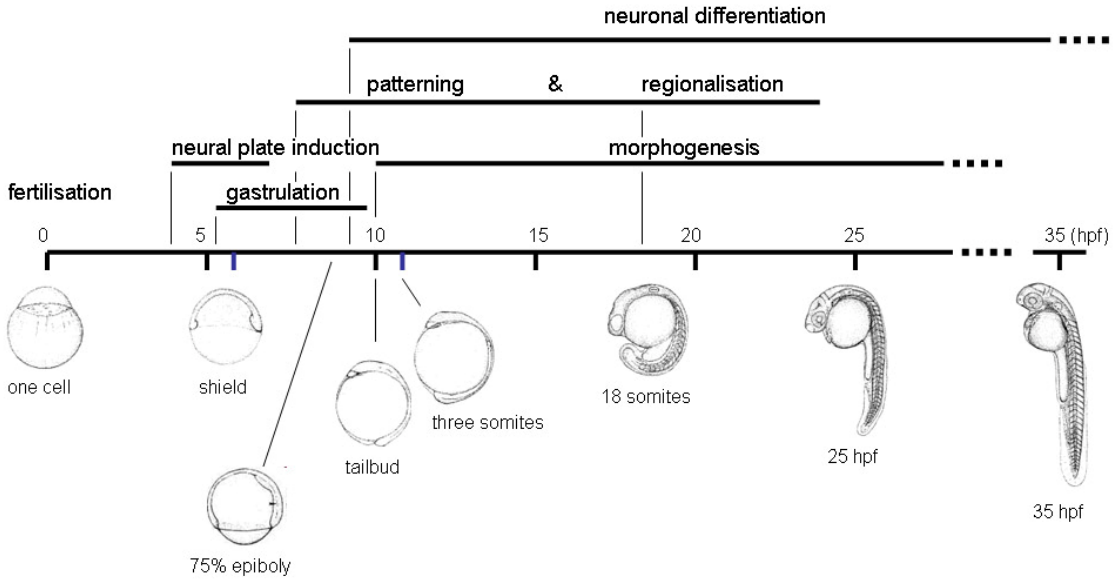

Figure 2. The development of the zebrafish embryo from fertilization until 1.5 days. Percent epiboly refers to the fraction of yolk cells that is covered by the blastoderm and it is used to stage the embryos in the first ten hours. After tailbud stage at 10 hours post fertilisation (hpf), the segmentation stage begins. Here the number of somites is used to determine the embryonic stage. Figure partially adapted from Kimmel (1995).

The neural plate consists of neuroepithelial cells, which divide symmetrically allowing them to increase in number (Alvarez-Buylla et al., 2001). These cells are multipotent and eventually give rise to all of the major cell populations in the CNS. After neural tube formation, neuroepithelial cells become radial glial cells (see figure 4). These cells have their cell body in the ventricular zone and long radial fibres extending from the internal surface to the pial (outer) surface of the neural tube. This cell type was once thought to guide neuronal migration along the radial fibres. However, it has recently been shown that radial glia are in fact embryonic neural stem cells. These cells divide asymmetrically, creating one radial glial cell and one neuron at each division. Radial glial cells later differentiate into ependymal cells that form the internal lining of the neural tube (Spassky et al., 2005), oligodendrocytes and ultimately to astrocytes (Malatesta et al., 2000). In the course of development radial glial cells disappear in mammals, however cells with astrocyte characteristics remain as neural stem cells in the adult brain (Alvarez-Buylla et al., 2001) (see also section '1.3: adult neurogenesis'). It is important to note that although during neural induction the whole neural plate adopts a generic neural fate, the neural plate and its derivatives do not differentiate homogenously later in development (Geling et al., 2003; Kimmel, 1993). Rather, some neurons differentiate early on in so-called 'proneural clusters', whereas some cells are kept undifferentiated in so called 'progenitor pools'. This maintenance of progenitor pools is crucial in order to allow the development of all cell types. Premature differentiation only allows the differentiation of early-born cell types and it disorganises the shape and cytoarchitecture of the brain (Kageyama et al., 2005).

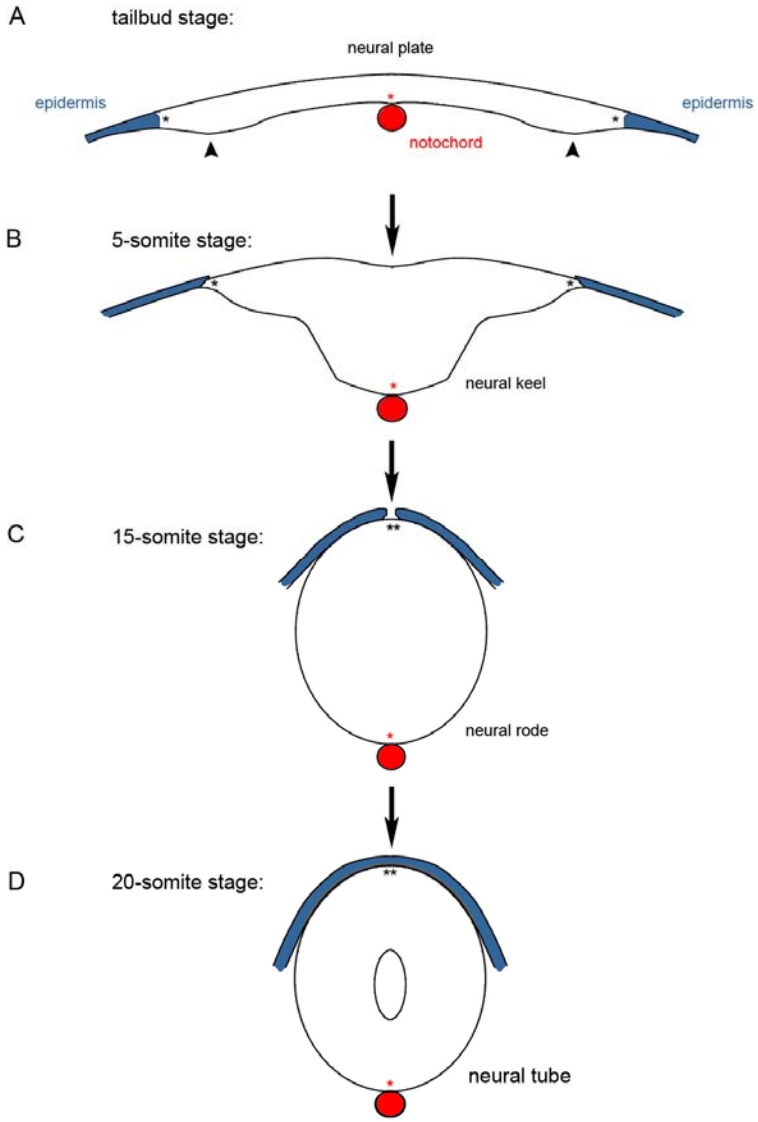

Figure 3. Morphogenetic processes during neurulation in zebrafish. Shown here are schematic cross sections through the early zebrafish embryo. Morphogenesis of the neural tube at tailbud (A), 5 somites (B), 15 somites (C) and 20 somites (D). Lateral neural plate bulges (marked by black arrows) converge towards the midline, which itself folds inward, leading to the formation of a compact neural keel/rod, which later forms a cavity. The notochord is positioned medially and is depicted in red. The black asterisks show that the originally lateral positions at the tailbud stage in (A) end up in dorsal positions, while the originally medial positions end up at the ventral most position (indicated by red asterisks). Modified from Papan and Campos-Ortega (1994).

The onset of neurogenesis in the zebrafish neural plate becomes apparent at late gastrulation with the expression of 'proneural genes', which commit the cells expressing them towards neurogenesis (Appel and Chitnis, 2002). The first proneural genes to be expressed encode transcription factors, such as the basic Helix-Loop-Helix (bHLH) proteins Neurogenin1 (Neurog1) and Achaete-scute1 (Ash1a) and the non-basic HLH transcription factor Coe2. Among the first neurons to differentiate in all species make up the ventrocaudal cluster (vcc), a basal cluster located at the diencephalic-mesencephalic junction (Chitnis and Kuwada, 1990; Mastick and Easter, 1996) and motor neurons of the hindbrain, which arise in rhombomeres two and four (Lumsden and Keynes, 1989). Other sites of neurogenesis include the spinal sensory neurons, spinal motorneurons and spinal interneurons, as well as in the trigeminal placodes and in the epiphysis and olfactory placodes. Figure 5 depicts the spatial pattern of proneural clusters and progenitor zones in the early zebrafish embryo. How these progenitor zones are maintained is a topic of this thesis, and it will be introduced in the following pages.

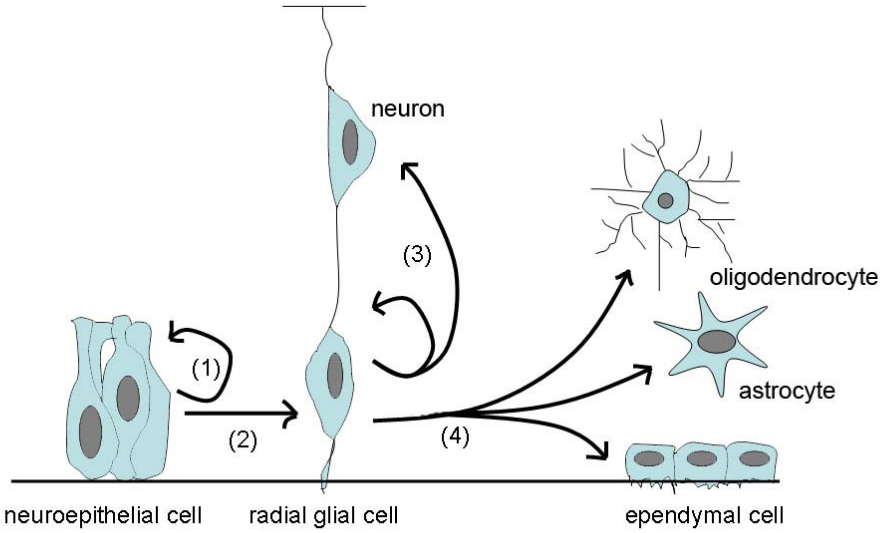

Figure 4. A simplified model of mammalian neural stem cell (NSC) evolution during development. Neuroepithelial cells divide symmetrically to increase in number (1), before forming radial glial cells (2). The radial glial cells divide asymmetrically forming one radial glial cell and one neuron from each division (3). Finally the radial glial cells form astrocytes, oligodendrocytes and ependymal cells (4). Adapted from Kageyama et al. (2005).

1.1.2 The delimitation of proneural fields by prepatterning factors

As discussed above, neurogenesis in the early vertebrate neural plate occurs at stereotyped loci, avoiding other areas of the neural plate, suggesting the existence of a prepattern of proneural/neurally-incompetent fields. Several transcription factors that promote neural fate immediately downstream of neural induction have been identified. These include members of the Sox, Gli, POU and Iroquois families. They are expressed in broad domains of the neural plate (Bainter et al., 2001; Bally-Cuif and Hammerschmidt, 2003). In zebrafish *iro1*, *iro7* and *pou5f1* are expressed across the presumptive midbrain and hindbrain areas and they are required for *neurog1* expression in their respective expression domains (Hauptmann and Gerster, 1995; Itoh et al., 2002; Lecaudey et al., 2001). When overexpressed, Iro1 and 7 are sufficient to induce ectopic *neurog1* expression within non-neural ectoderm (Belting et al., 2001; Itoh et al., 2002).

In addition, evidence from zebrafish and *Xenopus* shows that proneural fields are defined negatively, as domains that do not express active neurogenesis inhibitors. These domains include the anterior neural plate (prospective telencephalon, diencephalon and eyes), the longitudinal spinal cord stripes that separate the columns of sensory, motoneurons and interneurons and the midbrain-hindbrain boundary (mhb). These areas are characterized by the expression of neurogenesis inhibiting transcription factors – members of the Zic, Iro and Hairy/Enhancer of Split (Hairy/E(Spl)) families.

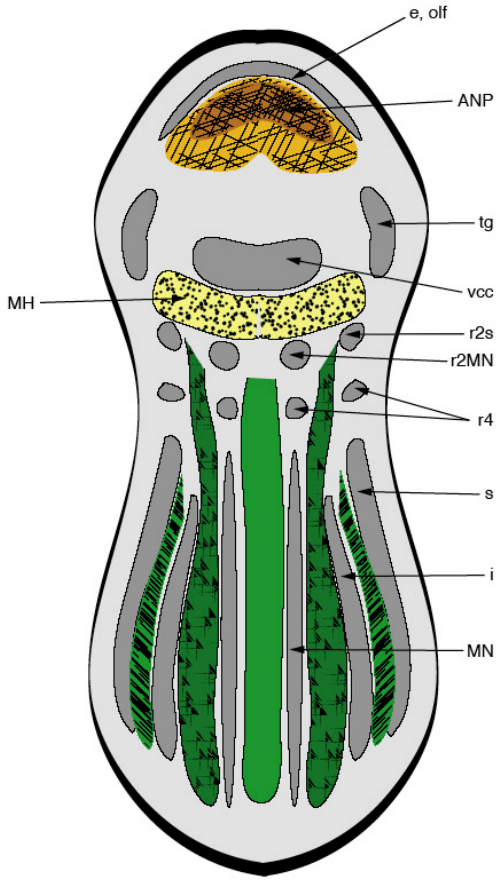

Figure 5. Schematic representation of the spatial pattern of neurogenic and progenitor zones in the zebrafish embryo (dorsal view of the early neural plate (3 somites), anterior to the top). The areas of neurogenesis ('proneural clusters') are depicted in grey. The progenitor zones are depicted in green. Abbreviations: eye = eye field; olf = olfactory neurons; r2 = sensory and motor neurons of rhombomere 2; r4 = sensory and motor neurons of rhombomere 4; MN = motorneurons; MH = midbrain-hindbrain boundary; tg = trigeminal ganglion neurons; vcc = ventro-caudal cluster; s = sensory neurons; i = interneurons.

In the following text I will first discuss the role of Notch and lateral inhibition in neurogenesis control within the proneural clusters, before moving on to a more detailed description of the role of the bHLH Hairy/Enhancer of Split (Hairy/E(Spl) factors in the active inhibition of neurogenesis in the progenitor zones.

Lateral inhibition selects neural progenitors within the proneural clusters

Although all cells within a proneural cluster have the potential to form neuroblasts and they all express low levels of proneural genes, only a limited number of cells undergo differentiation at any one time. The selection of the cells that remain as precursors depends on a process called lateral inhibition. In the process of lateral inhibition one of the cells is selected to form a neuroblast, while its neighbours adopt a non-neural fate. The cell that will become a neuroblast expresses elevated levels of proneural genes while the other cells down regulate proneural gene expression (Skeath and Carroll, 1992).

Lateral inhibition begins when a cell that is destined to become a neuron expresses Delta on its cell surface. This binds the Notch receptor on the surface of neighbouring cells. The binding of a Notch ligand allows γ-secretase to cleave the Notch intracellular domain (NICD) (figure 6). The NICD then translocates to the nucleus, where it forms a complex with the DNA-binding protein RBP-J (Nishimura et al., 1998), converting it from a repressor into an activator (Mumm and Kopan, 2000). RBP-J typically represses members of the Hairy/E(Spl)

family that repress neurogenesis, such as mouse *Hes1* and *Hes5*. However, when RBP-J forms a complex with NICD, this complex can instead induce *Hes1* and *Hes5* expression.

Following lateral inhibition, committed progenitors express increased level of Delta and proneural genes and further mechanisms are required to increase and/or maintain a high level of proneural gene expression in differentiating cells. The increased level of initial proneural genes causes the expression of a second set of proneural genes (for example *senseless* in *Drosophila* (Nolo et al., 2000) or *Hes6* in vertebrates (Bae et al., 2000; Koyano-Nakagawa et al., 2000) (see also section '1.1.10 Hes6 homologues in zebrafish'), which in turn further upregulate proneural gene expression. The proneural genes inhibit the expression of *E(Spl)* genes, as well as Notch signalling (Nolo et al., 2000) or they interfere at post-transcriptional levels with the inhibitory activity of bHLH factors activated by Notch signalling (Bae et al., 2000).

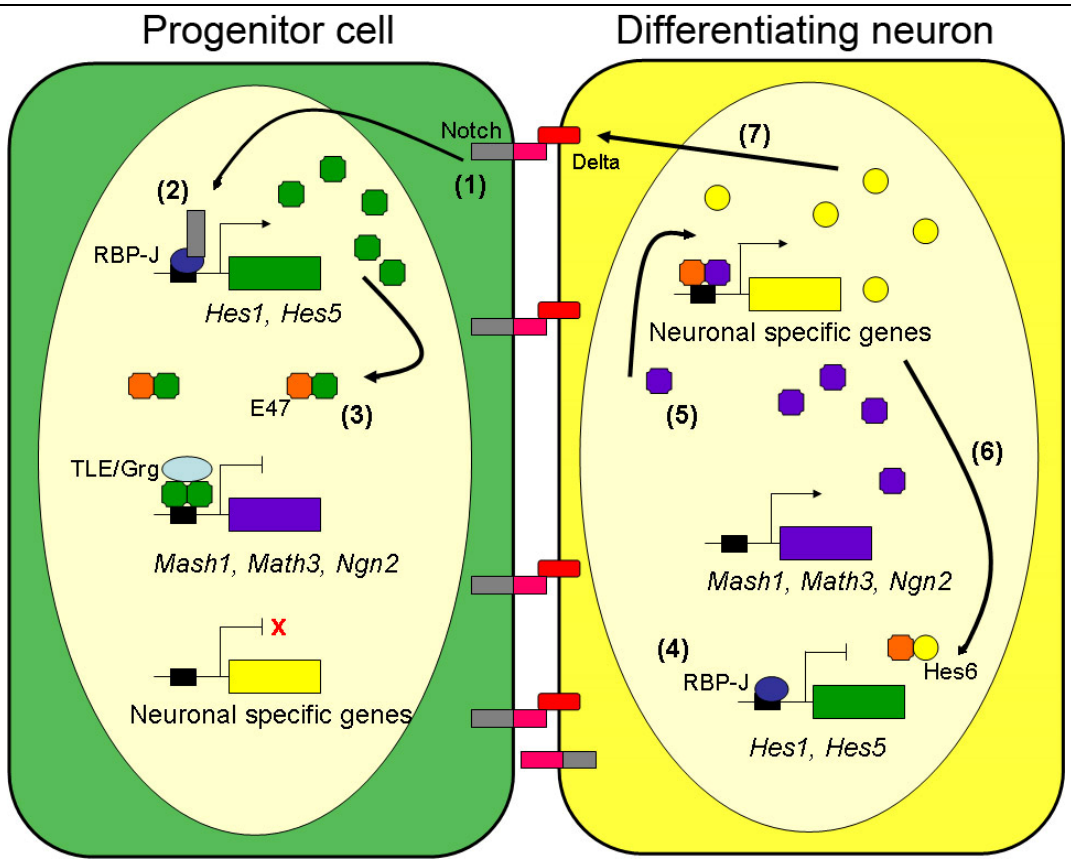

Figure. 6. Regulation of neural development by bHLH genes in mouse. 1) A progenitor cell expresses Notch, which is activated by Delta. 2) The notch intracellular domain (NICD) is released and transferred into the nucleus, where it forms a complex with RBP-J. This complex induces the expression of *Hes1* and *Hes5*. 3) Hes1 and Hes5 bind with E47, preventing it binding with Mash1, Math3 or Ngn2 and activating the expression of neuronal specific genes. 4) In differentiating neurons Notch is not activated and RBP-J represses the expression of Hes1 and Hes5. 5) Mash1, Math3 and Ngn2 bind with E47 and activate neuronal specific genes. 6) The neuronal specific genes induce expression of *Hes6*, which binds with Hes1 and Hes5 (adapted from Kageyama et al., 2005).

In zebrafish several neurogenic mutants that belong to the lateral inhibition cascade have been described: *deltaA* (Appel et al., 1999), *after eight* (mutation in *deltaD*) (Holley et al., 2000) and *deadly seven* (mutation in *notch1a*). All of these mutants show an increase in the number of primary neurons. The mildness of the phenotype is likely to be due to the redundancy of Notch and Delta paralogs (Chapouton and Bally-Cuif, 2004). Similarly, *mind bomb* mutants, which have mutation in a RING ubiquitin ligase that mediates Notch cleavage, show a neurogenic phenotype (Mind bomb is an ubiquitin ligase that is essential for efficient activation of Notch signalling by Delta). Again, similar to *Drosophila*, loss of function mutations in neurogenic genes, such as *neurog1* (neurogenin 1), lead to a decrease in the number of neurons in zebrafish (Cau and Wilson, 2003; Dornseifer et al., 1997; Geling et al., 2004; Golling et al., 2002). Notch signalling can be experimentally prevented, through the blocking of γ -secretase activity with the specific chemical blocker, DAPT. This leads to a loss of functional Notch signalling and a strong neurogenic phenotype due to the abolishment of lateral inhibition (Geling et al., 2002). In summary, lateral inhibition is necessary to assure that only a subset of progenitors within a neural cluster undergo neuronal differentiation and this mechanism is highly conserved between *Drosophila* and vertebrates.

1.1.3 The role of bHLH transcription factors in neurogenesis

As outlined above, neurogenesis is regulated by a balance between positive factors that promote neuronal differentiation and negative regulators that prevent differentiation from taking place. Many genes are necessary for the normal patterning of neurogenesis in the embryo, including a group of proneural genes that encode basic Helix-Loop-Helix (bHLH) transcription factors (Bertrand et al., 2002).

The bHLH proteins form a large superfamily of transcriptional regulators that are found in organisms from Arabidopsis to yeast to humans. They function in critical developmental processes, including sex determination and the development of the nervous system and muscles (for a review see Davis and Turner, (2001)). bHLH proteins can be classified into six major groups (A-F) (see table 1) based on evolutionary analysis and taking into account E-box binding, conservation of residues in the other parts of the motif and the presence or absence of additional domains (see Jones (2004) for a review). A focus of this thesis is the maintenance of progenitor cells via Hairy/Enhancer of Split factors. Phylogenetic comparisons suggest the existence of four major subfamilies within the Hairy/E(Spl) family (group E). They are Hairy, E(Spl), Hey and Stra13 (Iso et al., 2001) (see figure 7). In zebrafish at least 19 Hairy/E(Spl) proteins have been identified. A phylogram of the different teleost Hairy/E(Spl) proteins and *Drosophila* Hairy is shown in figure 8.

Table 1. bHLH proteins can be classified in six major groups (adapted from Jones, 2004).

Phylogenetic group	Description	Examples of classified proteins (family names)	Example mammalian protein	Function of example mammalian protein
A	Bind to CAGCTG or CACCTG	MyoD	Myf4	Myogenic: initiates myogenic programme in many cell types
		NeuroD	NDF2	Neurogenic: involved in terminal neurone differentiation
		Neurogenin	Neurog1	Promotion of early neurogenesis
B	Bind to CACGTG or CATGTTG	Mad	Mad1	Regulation of cell proliferation
		Myc	C-Myc	Cell proliferation and differentiation; oncogenic
C	Bind to ACGTG or GCGTG. Contain a PAS domain	Single-minded	Sim1	Neurogenic: regulation of midline cell lineage in the central nervous system
		Clock	Clock	Regulation of circadian rhythm
D	Lack a basic domain and hence do not bind DNA but form protein-protein dimers that function as antagonists of group A proteins	Emc	Id1	Myogenic and neurogenic: negative inhibition of DNA binding
E	Bind preferentially to N-box sequences CACGCG or CACGAG. Contain an orange domain and a WRPW peptide	Hairy/Enhancer of Split (E(Spl)	Hes1	Neurogenic: restricts differentiation of neurons from neural precursor cells
F	Contain an additional COE domain, involved in dimerisation and DNA binding	Coe (Col/Olf-1/EBF)	EBF1	Hematopoietic: essential for B-cell development

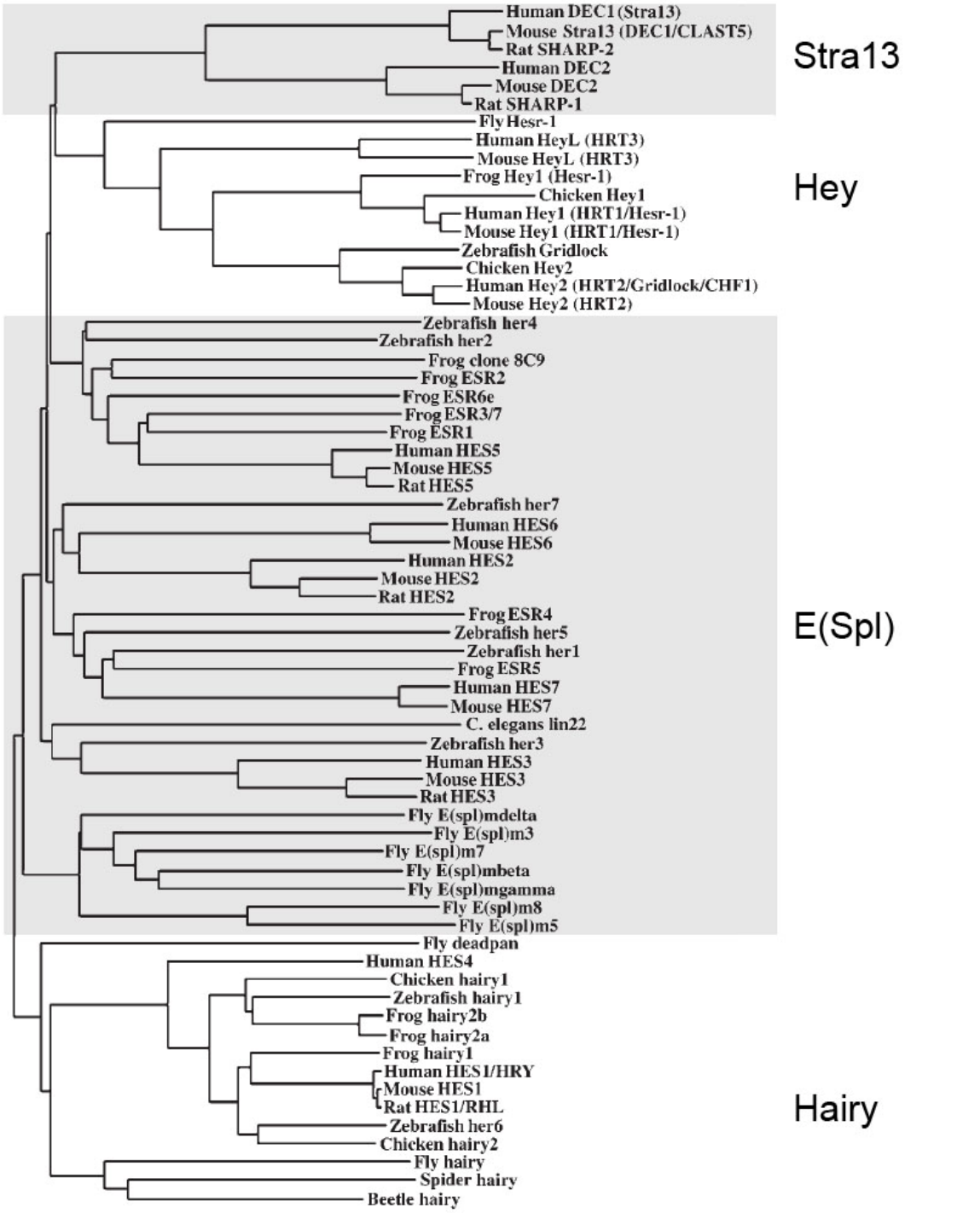

Figure 7. Phylogenetic tree for the Hairy/Enhancer of Split protein family, showing the separate subfamilies. See Figure 8 for a complete version of teleost Hairy/E(spl) factors. From Davis and Turner (2001).

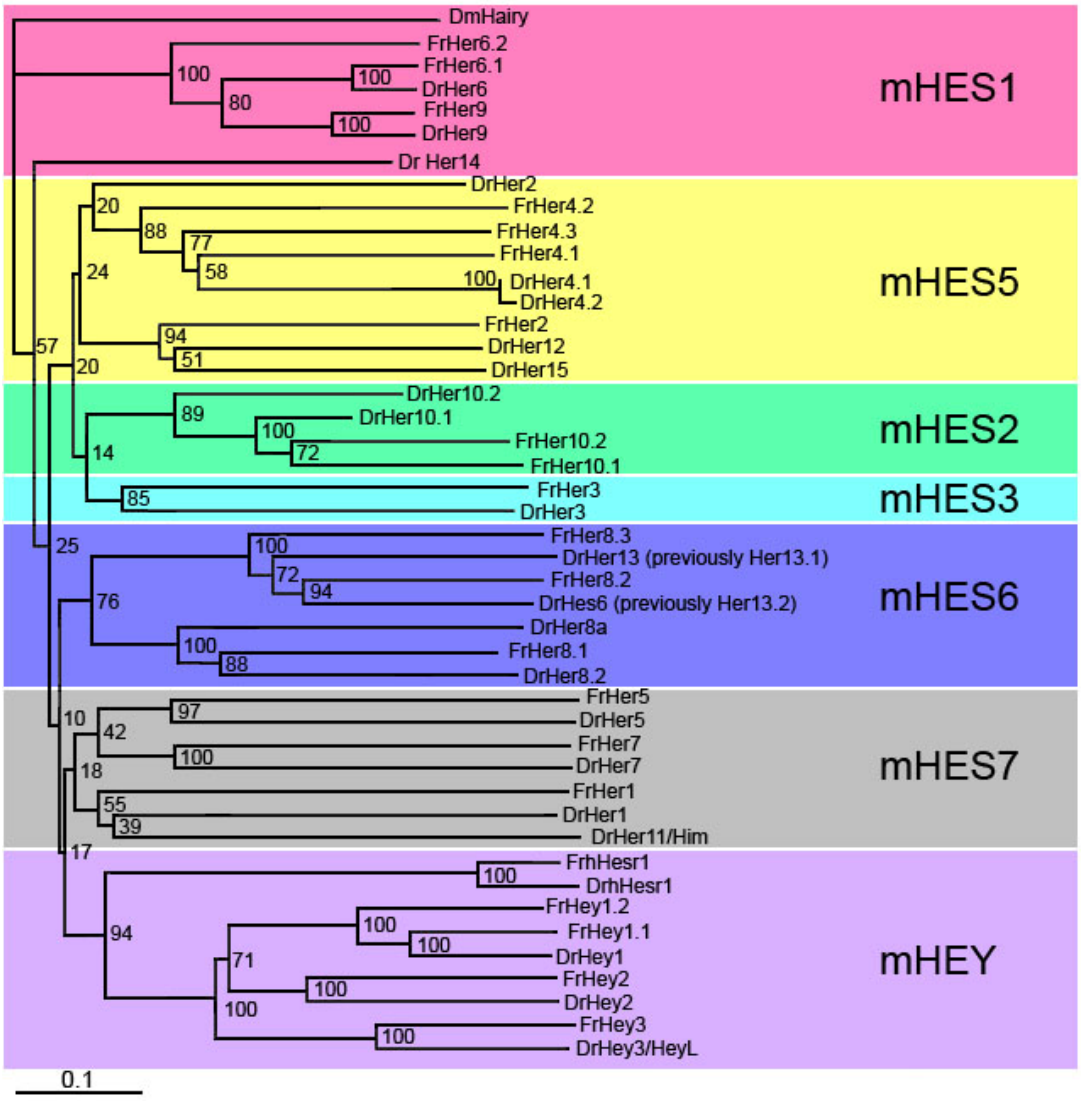

Figure 8. Phylogram of the different teleost Hairy/E(Spl) proteins and *Drosophila* Hairy. The colour coding indicates the closest mouse homologues. *Dm Drosophila melanogaster*, *Dr Danio rerio*, *Fr Takifugu rubripes*. Adapted from Sieger et al. (2004).

The original description of proneural clusters and the crucial genes involved in their maintenance and formation comes from *Drosophila* research, in particularly in the peripheral nervous system (PNS) (Fisher and Caudy, 1998). The bHLH factors involved in these processes can be separated into two classes: the repressor type genes and the activator type (also called proneural genes). Repressor type genes contain seven *Enhancer of split Complex* (*E(Spl)*) members and *deadpan*. Proneural genes include daughterless and four *achaete-scute* complex members (*achaete*, *scute*, *lethal of scute* and *asense*) and *atonal* (Fisher and Caudy, 1998).

In zebrafish, the first proneural genes expressed include Neurogenin1 (Neurog1) (Myers et al., 1986) and Achaete-scute1 (Ash1a) and Coe2 (Bertrand et al., 2002). Transcription of these genes is restricted to progenitors set to become neurons within the 'proneural clusters', which form the nuclei from which the primary neuronal network will arise (Stigloher et al.,

2008). Among the repressor type genes are members of the Hairy/Enhancer of Split (*Hairy/E(Spl)*) subfamily.

The regulation and role of *Hairy/E(Spl)*-related genes within proneural clusters

The Notch signalling pathway plays a major role in the regulation of transcription of many *Hairy/E(Spl)* genes. Overexpression of a constitutively active form of Notch activates the expression of a transiently transfected reporter based on the *Hes1* promoter, and this activation requires RBP-J binding sites (Jarriault et al., 1995). In addition, the expression of another family member, *Hes5*, is dependent on an intact Notch signalling pathway. *Hes5* expression is reduced or abolished in mice mutant for Notch1 and RBP-J (Barrantes et al., 1999). In *Xenopus* genes for several E(Spl)-related proteins are activated by Notch signalling, including ESR1, which is closely related to *Hes5* (Wettstein et al., 1997). *ESR1* can be blocked by a dominant negative ligand for Notch (Wettstein et al., 1997). Similarly, the zebrafish *E(Spl)*-like genes *her1* and *her4* are activated by Notch signalling (Takke and Campos-Ortega, 1999; Takke et al., 1999).

Overexpression of *Hes1*, *Hes3* or *Hes5* in the embryonic brain inhibits neuronal differentiation and maintains radial glia (Hirata et al., 2000; Ishibashi et al., 1994; Ohtsuka et al., 2001). In concordance, in *Hes1;Hes5* double knock-out mice, many radial glial cells are not maintained and prematurely differentiate in neurons (Ishibashi et al., 1995). Although many radial glial cells differentiate prematurely into neurons in *Hes1;Hes5* double knock-out mice, neuroepithelial cells and some radial glial cells are maintained, indicating that another Hes factor may compensate for the lack of *Hes1* and *Hes5*. Indeed, in *Hes1;Hes3;Hes5* triple knock-out mice, even neuroepithelial cells and practically all radial glial cells prematurely differentiate into neurons by E10.0, instead of differentiating into the later born cell types: namely later born neurons, oligodendrocytes, astrocytes and ependymal cells (Hatakeyama et al., 2004). Thus Hes1, Hes3 and Hes5 are crucial for the generation of cells in the correct numbers and their full diversity by preventing the differentiation of NPCs until later stages. Unusually, even in *Hes1*;*Hes3*;*Hes5* triple knockout mice the neuroepithelial cells are initially formed, signifying that the formation of NPCs is independent of *Hes* gene activities. They are, however, in absence of these *Hes* genes not maintained (Hatakeyama et al., 2004).

In zebrafish, Her4 (Appel et al., 2001; Haddon et al., 1998; Takke et al., 1999), Hes5 (Her15(Bae et al., 2005), Her2 and Her12 (Bae et al., 2005) are all E(Spl) factors expressed in proneural clusters in zebrafish. In *Drosophila* E(Spl) partakes in the lateral inhibition process. Similarly, in zebrafish Her4 has been shown to inhibit the expression of proneural genes (Takke et al., 1999).

1.1.4 Neurogenesis prevention outside of proneural clusters – the maintenance of progenitor pools

In addition to the regulation within the proneural clusters, additional negative regulatory factors act outside of these clusters in order to prevent proneural bHLH activity. These negative regulatory factors include bHLH factors that function as transcriptional repressors, such as *Hairy* in *Drosophila* (Van Doren et al., 1991) or HLH factors such as *Drosophila* extramachrochaete (Emc). Emc lacks a basic domain (it cannot bind DNA) and it forms non-functional dimers with bHLH factors leading to the prevention of DNA binding (Van Doren

et al., 1991). The elimination of these negative regulators results in ectopic neurogenesis, demonstrating that these negative regulators are imperative for the constraining of proneural bHLH activity to the proneural clusters.

In zebrafish, at the neural plate stage the proneural clusters are separated by large domains that no not express proneural genes (for reviews see Bally-Cuif and Hammerschmidt (2003)). These domains exhibit delayed differentiation, and cell tracing studies indicate that they will be only progressively recruited in early neurogenesis and/or will participate in later neurogenesis events (Stigloher, 2007). These domains are characterised by the expression of a specific set of transcription factors, including Zic, BF, Anf and Rx proteins (Bally-Cuif and Hammerschmidt, 2003), as well as a distinct set of *E(Spl)* genes in progenitor pools – which, to date, include zebrafish *her3*, *her5*, *her9* and *him*/*her11* (Stigloher et al., 2008) (see figure 9, A). *her5* is expressed from 70% epiboly onwards (see figure 2 for a description of early embryonic staging), in a domain of the neural plate that prefigures the early mhb, separating the ventrocaudal cluster (vcc) from the presumptive motor- and lateral neurons in rhombomere 2 (Mueller et al., 1996). *her3* is expressed starting at 30% epiboly in a coherent patch of cells within the dorsal region of the epiblast (Woo and Fraser, 1995). At 80% epiboly this first domain splits into two, which finally form by tailbud two longitudinal stripes (Hans et al., 2004). *her9* is expressed in longitudinal stripes (separating different types of spinal neurons), at the mhb and at the eye field (Bae et al., 2005; Leve et al., 2001). *him*/*her11* is expressed in a similar manner to *her5* at the mhb. Thus together these genes cover most of the domains of progenitor pools.

her3, *her5*, *her9* and *him/her11* exhibit functional similarities. Overexpression of these genes broadly inhibits *neurog1* expression, whereas loss of function causes ectopic expression at least in part of their expression domains (Bae et al., 2005; Geling et al., 2004; Hans et al., 2004; Ninkovic et al., 2005) (see figure 9, B). Thus these genes function in the suppression of proneural gene expression, although it is not yet known whether they target these genes directly.

The pattern of neural development displays similarities across all vertebrates. Mouse, zebrafish and chicken share prominent neuronal clusters in the forebrain, such as the nucleus of the medial longitudinal fascicle (vcc in zebrafish, interstitial nucleus of Cajal in mouse and chicken), and in all vertebrates rhombomeres 2 and 4 of the hindbrain differentiate earlier than others (Chedotal et al., 1995; Easter et al., 1994; Mastick and Easter, 1996). Therefore, at early embryonic stages, it appears that the alternation between areas of neurogenesis (proneural zones) and areas of neurogenesis inhibition (progenitor zones) along the neural tube is evolutionary conserved.

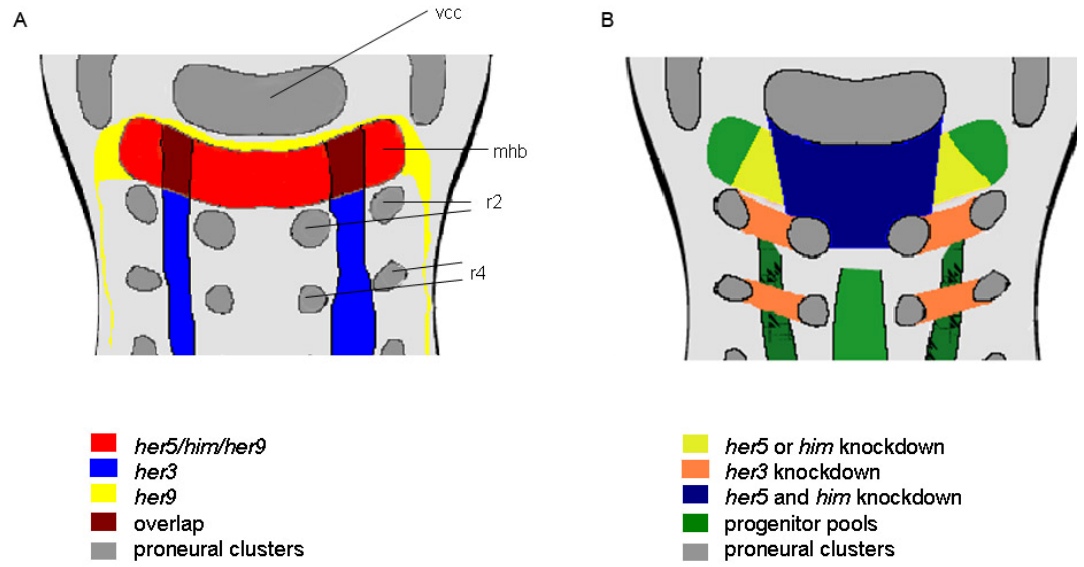

Figure 9. The knockdown of a subset of *her* genes causes ectopic proneural gene expression in part of their domains. Schematic of a dorsal views of the early zebrafish neural plate (three somite stage). Proneural clusters are depicted in dark gray. A) depicts the expression of *her3* (blue), *her9* (yellow) and *her5*/*him*(*her11*)/*her9* (red). B) The knockdown (using morpholino antisense oligos) of *her3* causes ectopic neuronal induction in rhombomeres 2 (r2) and 4 (r4) (orange). Single *her5* or single *her11* knockdown causes ectopic neurogenesis in the medial part of the mhb (blue). If both genes are knockdown simultaneously, the ectopic expression of proneural genes extends to include the lateral mhb (lime-green).

As described above, mouse Hes1, Hes3 and Hes5 are required to prevent premature neuronal differentiation in the developing neural tube (Hatakeyama et al., 2004; Hirata et al., 2001; Ishibashi et al., 1994). Recently, differences between the expression of *Hes1* at neurogenic zones and at boundaries, such as the mhb, the zona limitans, interrhombomeric boundaries and the roof and floor plates of the spinal cord, have been shown (Baek et al., 2006). In neurogenic zones, *Hes1* is expressed in a salt and pepper fashion, alternating with cells expressing *Mash1*, a proneural gene. In these areas *Hes1* also responds to lateral inhibition. In contrast, at the boundaries mentioned above, *Hes1* is expressed at high levels in all cells. This, and the fact that Hes1 can be activated in the absence of Notch in the mhb, suggests that Hes1 may play a role similar of that to the *her* genes - *her3*, *her5*, *her9* and *her11*.

1.1.5 Inhibitory factors function redundantly in order to secure progenitor pool maintenance

Premature differentiation of progenitor zones prevents the normal development of central nervous system structures. For example, premature differentiation of the anterior neural plate (ANP) in *Xenopus* prevents the expansion of the cerebral hemispheres (Ermakova et al., 1999), while early differentiation of the mhb impairs midbrain growth and the formation of some neural populations (Hirata et al., 2001). Therefore, neurogenesis inhibition appears to be essential for the maintenance of progenitor pools that allow the expansion and

diversification of the CNS. It appears that a number of functionally redundant pathways have evolved in order to secure this process.

One such example is from the mouse mhb. At late stages in mouse embryos, the mhb is maintained by the redundant actions of Hes1 and Hes3 (Hirata et al., 2001). These factors have been shown to inhibit neurogenesis in vivo (Ishibashi et al., 1994; Kageyama and Ohtsuka, 1999) and double knockouts for Hes1 and Hes3 exhibit premature neural differentiation preceded by the down-regulation of the expression of mhb identity markers (Hirata et al., 2001).

In zebrafish, *her5* and *her11* work together to prevent neurogenesis at the mhb. In isolation, each of these genes is necessary for the maintenance of neuronal inhibition across the medial aspect of the progenitor pool. However, if both genes are inactivated together, the ectopic neurogenesis is extended across the entire mhb progenitor pool (Ninkovic et al., 2005). Hence, her5_and *her11* work redundantly to repress neurogenesis across the mhb progenitor pool.

In another example in zebrafish *her3* and *her9* are expressed in the posterior inter-proneural domains, and they are required for its formation (Bae et al., 2005; Hans et al., 2004). Inhibition of Her3 and Her9 leads to ectopic expression of the proneural genes in part of the inter-proneural domains. However, if both are inhibited at the same time there is ubiquitous expression of proneural genes and the inter-proneural domains are not formed (Bae et al., 2005). This suggests the existence of parallel pathways securing neurogenesis inhibition in the posterior neural plate.

1.1.6 Notch-independent control of Hes factor expression

As described above, *Hes1* is upregulated in proneural clusters in response to lateral inhibition caused by the expression of Delta on a neighbouring, differentiating neuron. However, it appears that not all expression of *Hes1* is dependent on Notch signalling, and indeed *Hes1* expression occurs at early stages when *Notch* and *Delta* are not expressed. In addition, Hes1 has been shown to be regulated in a Notch-independent manner by c-Jun N-terminal kinase signalling in human endothelial cells (Curry et al., 2006). Thus, *Hes1* may act as an effector for Notch in the segmenting mesoderm and in proneural clusters, while acting in a Notch independent manner in particular progenitor pools.

In contrast to *Hes1* (and also *Hes5*), there is no evidence that *Hes3* expression is controlled by Notch signalling at all (Nishimura et al., 1998). Thus, it seems that Notch signalling is not a sole regulator of *Hes* gene expression; although it is still unknown which factors regulate *Hes* expression.

In zebrafish, *her15* (formerly *hes5*) expression is strongly reduced or abolished in the *mind bomb* mutant, as well as in DAPT treated wildtype embryos. In addition, expression of the Notch intracellular domain (NICD) strongly induces ectopic expression of *her15*. Likewise, *her4* expression is down-regulated by DAPT at early embryonic stages (Ozbudak and Lewis, 2008). This is consistent with *her15* and *her4* being targets of Notch signalling. In contrast, *her5* expression is not affected by DAPT and it is down-regulated, rather than upregulated upon NICD misexpression (Geling et al., 2004). *her3* is also suppressed by NICD (Hans et al., 2004), whereas *her9* expression remains unaffected (Bae et al., 2005). *her15* and *her4* are

expressed alternatively with proneural genes such as *neurog1*, in a salt and pepper fashion in the proneural clusters of developing embryos, whereas *her3*, *her9* and *her5* are expressed outside of the proneural clusters in progenitor pools. This indicates that at least at early embryonic stages the expression of *her3*, *her9*, *her5* and possibly other *her* genes outside of the proneural clusters is not dependent on Notch.

1.1.7 Patterning of the neural plate/tube

After, and partially during neural induction, is the establishment of neural plate/tube patterning. It is important to note that the processes of neural induction and anterior/posterior patterning can not be clearly separated in time and space or by the factors that are involved. They are rather happening in concert with each other. Here I will focus on patterning along the anterior-posterior axis, as along this axis several local signalling centres have been identified that influence neural plate patterning and correlate with specific neurogenesis status. Examples of these signalling centres are the anterior border of the neural plate, the zona limitans intrathalamica and the isthmic organiser (IsO) at the mhb. Importantly, these organisers often coincide with progenitor pools.

Here I will focus on the patterning of the midbrain-hindbrain boundary (mhb), as one of the focuses of this thesis is on *her5* – a gene expressed in this area.

Patterning of the midbrain-hindbrain domain

The midbrain-hindbrain domain, that is the midbrain and anterior hindbrain, can be morphologically identified at early somitogenesis stages as consisting of the mesencephalon and the metencephalon. The mesencephalon generates all midbrain structures and the metencephalon subdivides later into rhombomeres 1 and 2 of the hindbrain (Marin and Puelles, 1994; Martinez and Alvarado-Mallart, 1989). The midbrain-hindbrain boundary is formed at the border between the mesencephalon and the metencephalon, as seen from a ventral view. Elegant transplantation studies performed on chick embryos have shown that if tissue at the mhb is removed and relocated to a new position in the brain, it can induce midbrain or cerebellum structures (reviewed in Wassef and Joyner (1997)).

The embryonic mhb is at the same time a crucial source of progenitors as well as hosting a secondary neural plate organiser, the isthmic organiser (IsO). This organiser plays a critical role in the patterning of the midbrain-hindbrain domain, giving rise to many key neural structures, such as alar parts of the midbrain (superior and inferior culliculi or tectum), the ventral midbrain nuclei, anterior hindbrain nuclei and the cerebellum (Zervas, 2004; Partanen, 2007).

Key molecules functioning in the formation of the mhb are Fgf8, Pax2.1, Wnt1, Her5, Eng2 and Eng3. Fgf8 is a signalling molecule that is secreted by the organiser to control the development of the midbrain and the anterior hindbrain. If a bead coated with recombinant Fgf8 is implanted in the posterior diencephalon of chick embryos, *Fgf8*, *Wnt1* and *Eng2* expression is enduced in the neighbouring cells. This completely transforms the posterior

diencephalon into the midbrain (Crossley et al., 1996). In zebrafish, the loss of Fgf8 function in the mutant *Ace* results in loss of the mhb, cerebellum and tectum (Brand et al., 1996).

Two major regulatory steps can be distinguished in midbrain-hindbrain development: the establishment phase, in which mhb genes are expressed independently of each other, and the maintenance phase, in which the expression of mhb factors becomes dependent on each other. During the establishment phase, the transcription factors *otx2* and *gbx1* define the prospective mesencephalic and metencephalic domains, respectively (Broccoli et al., 1999) (see figure 10). At least three parallel pathways (Pax, Wnt and Fgf) are independently activated in response to these patterning signals at the Otx2-Gbx1 interface. The maintenance phase starts at around the mid-somitogenesis stage (Lun and Brand, 1998). At this phase the expression of *pax2.1*, *wnt1* and *fgf8* becomes mutually dependent – if any one of these genes is disrupted, the continued development of the mhb is prevented (Rhinn and Brand, 2001).

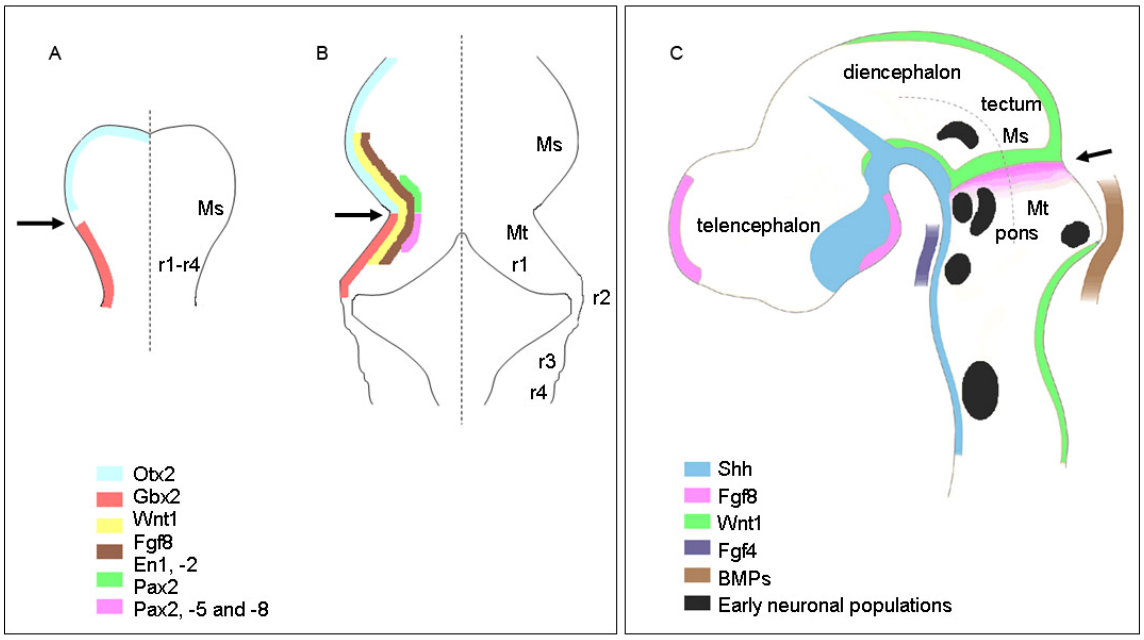

Figure 10. Development of the midbrain-hindbrain boundary (mhb). Schematic views of the mouse embryonic neural plate and the mouse neural tube. A and B) dorsal views of the mouse embryonic neural plate at A) 0 somites and B) E10 stage. At the end of gastrulation (0 somites), the neural plate is subdivided into an anterior domain that expresses *Otx2* and a posterior domain that expresses *Gbx2*. The expression of both genes meets at the presumptive midbrain-hindbrain boundary (mhb) (black arrow), and they form decreasing gradients from the mhb in opposite directions. At E10 the *Otx2-Gbx2* border identifies the mhb (black arrow). The expression of *Wnt1* and *Fgf8* becomes restricted to narrow rings encircling the neural tube on either side of the boundary. At this stage *En2*, *Pax5* and *Pax8* are still expressed broadly across most of the midbrain-hindbrain domain. C) Schematic view of the mouse neural tube at E11, anterior to the left. The position of the mhb is indicated by a black arrow. Secreted factors such as Fgf8, Shh, Fgf4 and members of the BMP family control neuronal identities. En, engrailed; Fgf8, fibroblast growth factor 8; Gbx2, gastrulation brain homeobox 2; Ms, mesencephalon; Mt, metencephalon; Otx2, orthodenticle homologue 2; Pax, paired box; r, rhombomeres; hatched line, axis of symmetry. Adapted from Wurst and Bally-Cuif (2001).

The maintenance of the boundary cells is essential for the development of region-specific neurons. In the mouse, the mhb expresses *Hes1* and *Hes3* and in the absence of these genes the mhb cells prematurely lose *Wnt1* and *Fgf8* expression, and they differentiate into ectopic neurons (Hirata et al., 2001). As a consequence, the midbrain and hindbrain neurons fail to be properly specified. For example, oculomotor and trochlear nuclei and the dopaminergic neurons of the midbrain and locus ceruleus neurons of the hindbrain are absent in *Hes1*:*Hes3* double knockout embryos (Hirata et al., 2001).

her5 is the earliest known gene to be expressed at the presumptive midbrain hindbrain boundary in the zebrafish. *her5* expression coincides with a number of markers that define the mhb (Belting et al., 2001; Lun and Brand, 1998; Reifers et al., 1998; Reim and Brand, 2002). However, ectopic *her5* expression (caused by the repeated heat shocking of a transgenic line with *her5* under the control of a heat shock promoter) does not appear to effect the expression of mhb patterning markers (*iro1, iro7, pax2.1, eng2, eng3*) or mhb activity markers (*wnt1, fgf8*) (Geling et al., 2005). Similarly, *her5* knockdown using morpholinos also did not produce changes in expression in the genes mentioned above (Geling et al., 2005). However, *her5* is responsible for the maintenance of the progenitor zone at the mhb. Hence, morpholino knockdown of *her5* results in the co-expression of mhb patterning markers (e.g. *pax2a* (formerly *pax2.1*)) and *neurog1*, a combination not seen normally (Geling et al., 2005).

Loss of Hes1 and Hes3 function also leads to a loss of the expression of many mhb genes, and consequently loss of mhb activity (Hirata et al., 2001). This is reminiscent of the function of *her5* in zebrafish (Geling et al., 2005).

1.1.8 Regulation of neural cell fate by *Hes* factors at later stages

At later stages of development *Hes1* and *Hes5* are transiently expressed by astrocytes in the developing brain (Nakashima et al., 2001; Wu et al., 2003) as well as in Müller glial cells in the developing retina (Furukawa et al., 2000; Hojo et al., 2000). Overexpression of Hes1 and Hes5 at later stages increases the number of astrocytes in the brain and Müller glial cells in the retina (Furukawa et al., 2000; Hojo et al., 2000; Ohtsuka et al., 2001; Takatsuka et al., 2004). In addition, the production of Müller cells is decreased in knockout Hes1 and Hes5 mice. This indicates that at least some Hes factors have a second role in development in addition to NPC maintenance – namely they are capable of altering the choice of neuronal versus glial cell fate at later stages. Interestingly, the proneural bHLH gene *Neurogenin1* (*Neurog1*) also performs two roles: the promotion of neurogenesis and the inhibition of gliogenesis (Sun et al., 2001). Neurog1 sequesters the CBP-Smad1 transcriptional complex away from the glial-specific promoters and recruits the complex to neuronal-specific promoters, so that neurogenesis is promoted at the expense of other fates. In addition, inactivation of the proneural genes *Mash1*, *Ngn2* and *Math3* prevents neurogenesis while promoting gliogenesis (Nieto et al., 2001). Thus, it is probable that the suppression of proneural genes marks one of the key mechanisms for *Hes* factor induced neurogenesis.

1.1.9 The structure of bHLH proteins

The structure of bHLH proteins is closely linked to their function. The bHLH domain is approximately 60 amino acids long. It contains a basic region, responsible for DNA binding (b), followed by two α -helices separated by a loop region (HLH) (Ferre-D'Amare et al., 1993) (figure 11, A). The HLH domain is responsible for dimerisation, allowing the formation of complexes with the same protein, or different members of the same family (see figure 11, B), while the basic region is required for the HLH dimers to make specific contacts with DNA (Blackwell et al., 1993; Ferre-D'Amare et al., 1993; Murre et al., 1989). Noticeably, a proline residue is conserved in the middle of the basic domain of all Hes factors as well as in that of *Drosophila* Hairy and E(spl) proteins. It is thought that this proline residue is involved in the specificity of target DNA sequences, although the exact significance of this conservation has not yet been determined. Hes factors are also characterised by a WRPW motif at their C-terminus and an orange domain (see below for details) (Dawson et al., 1995).

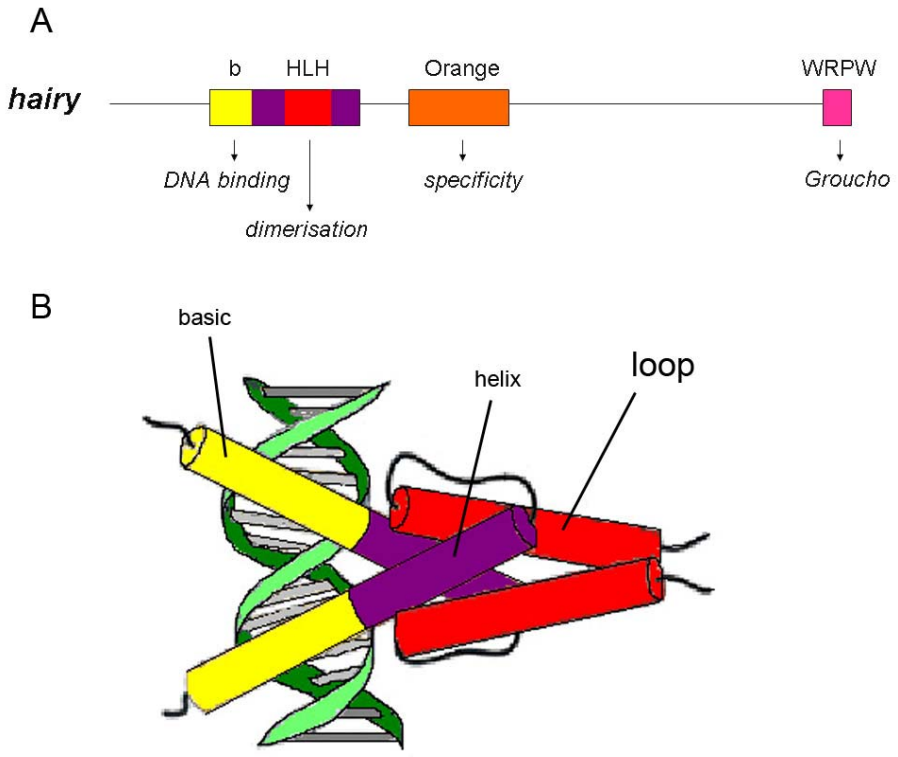

Figure 11. bHLH proteins all contain a basic Helix-Loop-Helix domain. A) Schematic drawing showing the protein domains of Hes proteins. The bHLH and Orange domains are present in all family members. These proteins usually end with WRPW, but in some cases with one or two additional amino acids. For example zebrafish Her7 ends with an additional proline. B) Schematic representation of the structure of a bHLH dimer bound to DNA. The two a-helices of both partners bind to make a four-helix bundle (adapted from Bertrand et al., (2002)).

1.1.10 Hes6 homologues in zebrafish

Hes6 is the closest mouse orthologue to the zebrafish genes *her8a*, *her13.1* and *her13.2* (see figure 8). A unique feature with respect to other members of the bHLH family is that the loop of *Hes6* is four to five amino acids shorter than usual (Bae et al., 2000). These proteins are characterised by a shortened loop – a feature also conserved in other fish species such as medaka and the pufferfish (Gajewski et al., 2006). The loops of Her13 proteins are five amino acids shorter, whereas Her8 proteins are 3 amino acids shorter than other members of this family (Gajewski et al., 2006). Hes6 has an unusal role in neural development, compared to other Hes family members, in that it promotes neurogenesis, rather than inhibiting it (see figure 12). Hes6 antagonizes Hes1 function by two mechanisms. Firstly, Hes6 inhibits the interaction of Hes1 with its transcriptional corepressor Gro/TLE. In addition, it promotes proteolytic degradation of Hes1 (Gratton et al., 2003). Mutational analysis has shown that loop-length is critical for the specific functions of *Hes1* and *Hes6* (Bae et al., 2000). An insertion of five amino acids into the loop of *Hes6* (Hes6ins) repressed the transcription of a luciferase gene under the control of the N box. In contrast, the removal of five amino acids from *Hes1* rendered it unable to repress the transcription of the same construct (Bae et al., 2000).

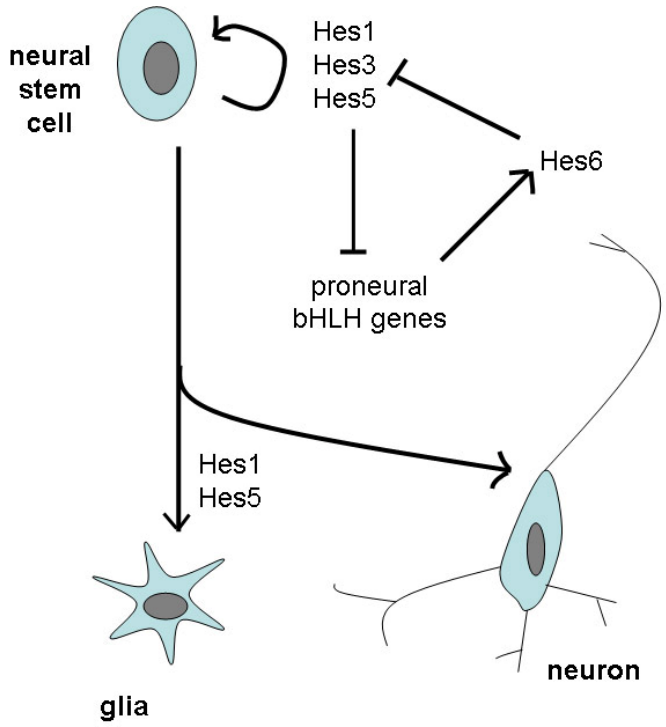

Figure 12. The role of *Hes6* in neural development. The bHLH proteins Hes1, Hes3 and Hes5 repress proneural bHLH gene expression and maintain neural stem cells. Proneural bHLH genes induce Hes6, which inhibits Hes1 and promotes neuronal differentiation. Cells that express Hes1 and Hes5 finally become glial cells (adapted from Kageyama et al. (2005)).

1.1.11 Mechanisms of transcriptional control by Hairy/E(Spl) proteins

Hes factors repress transcription by several mechanisms (see figure 13, A). One mechanism is 'active repression' mediated by the interaction between the corepressor Groucho and the WRPW motif at their carboxy-terminal. Groucho interacts with this WRPW motif and recruits histone deacetylase Rpd3, thereby modifying the chromatin structure (Grbavec and Stifani, 1996; Paroush et al., 1994).

For some interactions the WRPW appears to be dispensable. For example, *Drosophila* Hairy protein without the WRPW motif can still repress ectopic activation of *Sex-lethal* by the Scute bHLH protein (although this does not happen under endogenous conditions (Dawson et al., 1995). In the same way, a zebrafish Her4 protein without the WRPW motif can still repress neurogenesis in zebrafish embryos (Takke et al., 1999). This leads us to another method of repression: dominant-negative regulation (see figure 13, B). Most bHLH factors, such as Mash1, bind to the E-box (CANNTG) in order to activate gene expression. Repressor bHLH factors, such as Hes1 and Hes5, have been shown to form non-functional heterodimers with activator bHLH proteins, thereby inhibiting their activity (Akazawa et al., 1992; Sasai et al., 1992). The ability to bind E-boxes raises the possibility of a third mechanism of transcriptional repression, in which repressor bHLH factors compete with activator bHLH factors at binding sites (Davis and Turner, 2001) (see figure 13, C). In *Drosophila*, an optimal E-box binding site for E(Spl) proteins is also an optimal binding site for heterodimers of the *Drosophila* Daughterless and Lethal of scute bHLH activators, and these proteins compete for this site in *in vitro* DNA binding assays (Jennings et al., 1999). Such a competition for bHLH binding sites remains to be confirmed in vertebrates, but the ability of HES2 and other vertebrate proteins to bind to E-boxes suggests that this takes place.

Most HLH proteins bind to the E box (CANNTG) in the promoter region of their target genes (Blackwell and Weintraub, 1990). However, *Hes* factors preferentially bind to different target sequences than other bHLH factors, such as the class C site (CACG(C/A)G) or the N box (CACNAG) (Akazawa et al., 1992; Ohsako et al., 1994; Sasai et al., 1992).

Role of the orange domain in transcriptional repression

The Orange domain is an important functional domain that confers specificity among members of the Hairy/E(Spl) family. It is thought to consist of two amphipathic helices. Its importance and functionality was first described in *Drosophila* Hairy and E(spl) proteins (Dawson et al., 1995; Giebel and Campos-Ortega, 1997). *Drosophila* Hairy protein can prevent activation of *Sex-lethal* gene by the activator bHLH protein Scute (Parkhurst et al., 1990). In contrast, the E(spl) m8 protein can not, (Dawson et al., 1995) demonstrating that the specificity for the Scute inhibition is determined by the Orange domain. In vertebrates, the Orange domain has been shown to mediate transcriptional repression. Castella et al. (2000) reported that the HES1 Orange domain is necessary for HES1 to inhibit the activation of the *p21* promoter by MASH1 and E47.

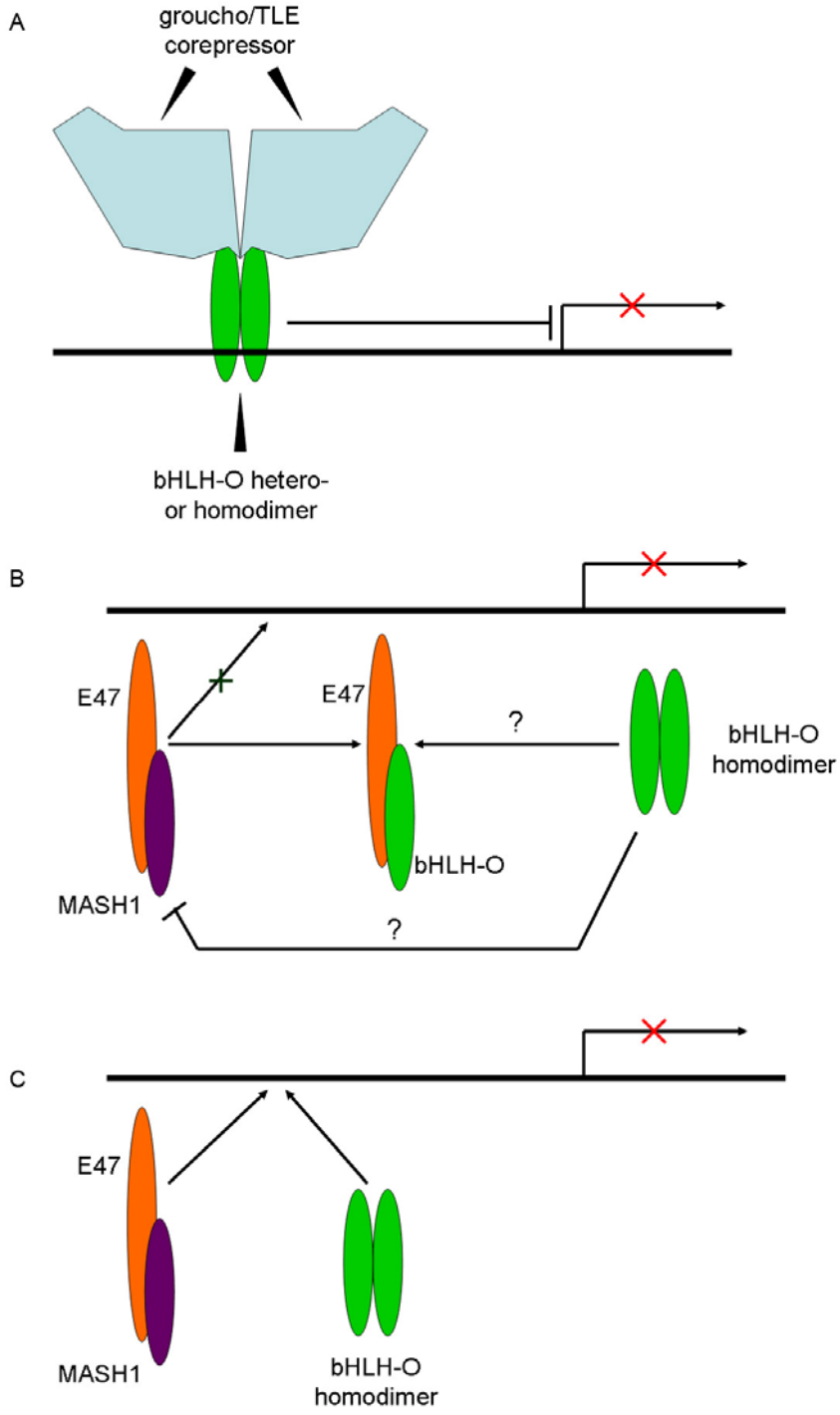

Figure. 13. Hairy/E(Spl) proteins repress transcription through various mechanisms. (A) A bHLH-O homo- or hetero-dimer recruits corepressors (in this case Groucho/TLE proteins) to target the promoters at specific binding sites. (B) Vertebrate Hairy/E(Spl) proteins inhibit reporter activation by activator bHLH heterodimers, such as MASH1-E47, and interact directly with bHLH activator proteins in vitro. (C) Hairy/E(Spl) proteins compete with activator bHLH proteins for the same DNA-binding sites (adapted from Davis and Turner, 2001).

The identification of dimerisation-partners: yeast-two-hybrid assay (y2h)

As outlined above bHLH proteins operate through the recruitment of other proteins in order to carry out their roles as either repressors or activators of transcription (see also table 2 for a complete list). Many heterodimer pairs of bHLH proteins have been isolated using y2h, a molecular biology technique to discover novel protein-protein interactions. The test is based on the activation of a downstream reporter gene, through the binding of a transcription factor to an upstream activating sequence (UAD). This transcription factor is split into two separate parts, called the binding domain (BD) and the activating domain (AD). The BD binds to the UAS and the AD is responsible for the initiation of transcription (Young, 1998). The protein or a fragment of the protein of interest is normally fused to the BD, while a library of known or unknown proteins is fused to the AD. These are subsequently cloned into an appropriate vector and then incorporated into the chosen screening organism. Once incorporated, if the bait and prey proteins bind, the AD and the BD of the transcription factor are indirectly connected and the transcription of the reporter gene can occur.

Y2h screens are now a very popular method of gaining a first impression of which partners interact with a protein of interest. The main disadvantage of the y2h system is a high rate of false positives and false negatives. Indeed the false positive rate has been estimated to be as high as 50% (Deane et al., 2002). The reasons behind this high error rate arise from the method itself: the assay investigates the interaction between (i) overexpressed (ii) fusion proteins in the (iii) yeast (iv) nucleus. Each one of points i-iv alone can give false results. Overexpression can result in non-specific interactions. In addition, some proteins may interact in yeast that are not physically in the same cell of the organism of interest. False negative results may arise when the protein is not correctly modified in yeast. However, despite these problems, y2h data is shown to be of similar quality to other methods, such as coaffinity purification followed by mass spectrometry (Yu et al., 2008). Furthermore, the disadvantages can be controlled for through further experiments, such as double expression analysis and co-immunoprecipitation studies.

As a basis for this study a yeast-two-hybrid screen was performed for us (Hybrigenics, France), using a fragment of the zebrafish Her5 protein as bait. It this way we identified novel potential interaction partners of Her5, in order to further our knowledge of the mode of action of Her5.

Table 2. Summary of protein-protein interactions of Hes proteins. Abbreviations: H1, helix 1; co-IP, co-immunoprecipitation; GST, GST pull-down assay; M2H, mammalian two-hybrid assay; Or, Orange domain; Y2H, yeast two-hybrid assay. Adapted from Fischer and Gessler (2007).

Interaction partner	Hes/Hey protein	Interacting Hes/Hey domain	Method	Comments	References
Homo/Heterodimers					
Hes1	Hes1	bHLH-Or	GST, co-IP, y2h		Iso et al. (2001)

Hey1,2	Hes1	bHLH (Or stabilizes)	GST, co-IP, y2h	Stronger than homodimers	Iso et al. (2001)
Hes6	Hes1	ND	co-IP	Repression of Hes1 activity	Bae et al. (2000)
Helt	Hes5 (not Hes1)	(Orange	co-IP (of Helt)		Nakatani et al. (2004)
Other HLH factors					
E47 E2-2	Hes1,5	ND	co-IP, M2H	Repression of transcriptional activity	Akazawa et al. (1992)
Id1,2,3,4	Hes1	ND	co-IP, M2H	Sequestration	Jogi et al. (2002)
ITF1,2	Hes1	bHLH	GST, y2h		Leimeister et al. (2000)
Mash1 (Ascl1)	Hes5	ND	co-IP	Repression, sequestration	Akazawa et al. (1992)
Ptf1-p48	Hes1	ND	GST, co-IP, y2h	Repression of transcriptional activity	Ghosh and Leach (2006)
Other transcription factors					
c-myb	Hes1	ND	co-IP	Repression of transcriptional activation of CD4 promoter	Allen et al. (2001)
GATA1	Hes1	ND	GST, co-IP	Represses GATA1 activity, but not DNA-binding capacity.	Ishiko et al. (2005)
RBPJκ	Hes1	bHLH (H1)	co-IP	Repression of transcriptional activity	King et al. (2006)
Runx2 (Cbfa1)	Hes1	C-terminus (not WRPW)	GST, co-IP, y2h	Enhances Runx2 activity, interferes with TLE1 and HDAC1 recruitment	McLarren et al. (2000)
Runx1 (Cbfa2)	Hes1	ND	GST, co-IP		McLarren et al. (2000)
Sox10	Hes5	ND	co-IP	Repression, sequestration	Murata et al. (2005)
STAT3 JAK2	Hes1,5	bHLH-Or	co-IP	Promotes STAT3 phosphorylation and nuclear translocation	Kamakura et al. (2004)
Transcriptional cofactors					
TLE1,2,3,4	Hes1,5,6	WRPW	GST,	Function as a	Ross et al.

			co-IP, y2h	corepressor	(2006); Fisher et al. (1996); Ju et al. (2004)
SIRT1	Hes1	bHLH	GST, co-IP	Augments repression capacity	Takata and Ishikawa (2003)
HDAC1	Hes1	ND	co-IP		Shen and Christakos (2005)
CBP	Hes1	ND	co-IP	Turns Hes1 into transcriptional activator	Ju et al. (2004)
Others					
pRB	Hes1	ND	co-IP	Enhances Runx2/Hes1 activity	Lee et al. (2006)
Ubiquilin 1	Hes1	ND	M2H		Persson et al. (2004)

1.2 Mechanisms of Addiction

Drug addiction can be broadly defined as a pathological state characterized by compulsive drug seeking and drug use, in spite of adverse consequences (Hyman and Malenka, 2001). At the addictive stage the drug becomes the primary source of positive emotions, at the expense of activities leading to natural rewards, such as food gathering and mating. Drug addiction occurs over three temporally sequenced stages: 1) acute drug effects, 2) transition from recreational use to pattern characteristic of addiction and 3) end-stage addiction, which is characterized by compulsive drug-seeking, reduced pleasure from biological rewards and a high probability of relapse, even after long periods of abstinence (Kalivas and Volkow, 2005).

Before addiction has occurred, drug cravings are produced through the release of dopamine in the nucleus accumbens (NAc). During the transition from drug use to addiction, the release of dopamine becomes unnecessary to produce cravings. Instead, dopamine transmission decreases, while hyperactivity in the orbitofrontal cortex contributes to the cravings. Addiction occurs when drug-seeking behaviour is exhibited and it is also defined by a persistent vulnerability to relapse, even after long periods of abstinence (Hyman, 2001; Wise, 2000). These changes are thought to be the result of neuroplastic changes in response to drug use and this is thought to be relatively permanent (Kalivas and Volkow, 2005).

Neuroplasticity in glutamatergic projections appears to be a major result of repeated drug exposure. Glutamate transmission is altered through an increase in the presynaptic release of glutamate and also through an increased response to glutamate (Jones and Bonci, 2005; Kalivas and Volkow, 2005). The increase in response to glutamate is facilitated through an increase in the expression of the two major glutamate receptors – NMDAR and AMPAR – on the cell surface. This type of synaptic plasticity results in long term potentiation (LTP) –a strengthening of the connections between two neurons.

In addition to producing compulsive use, psychostimulants and numerous other drugs of abuse can produce tolerance and sensitisation. Tolerance is defined as a decrease in the affect of a drug despite a constant dose, or a need for increased dosage to maintain a stable effect ((Hyman and Malenka, 2001). Some drugs, of which amphetamine and cocaine are the best examples, can produce sensitisation (enhancement) of some responses to the drug (Anagnostaras and Robinson, 1996; Kalivas and Stewart, 1991).

1.2.1 Mechanisms of Reward

The chemical structures of drugs of addiction vary, and along with them the initial molecular targets in the brain. For example, opiates, such as morphine, are agonists of opioid receptors, whereas cocaine inhibits the nerve terminal transporters for dopamine and other monoamine neurotransmitters (Nestler, 2001). Despite all of these differences, drugs of addiction share properties in common. Firstly, they share, along with natural rewards such as food and sex, the ability to increase dopamine transmission in the mesocorticolimbic system. A second common feature is that drugs of abuse influence neuronal plasticity in brain pathways related to motivation and reward (Wolf, 2002).

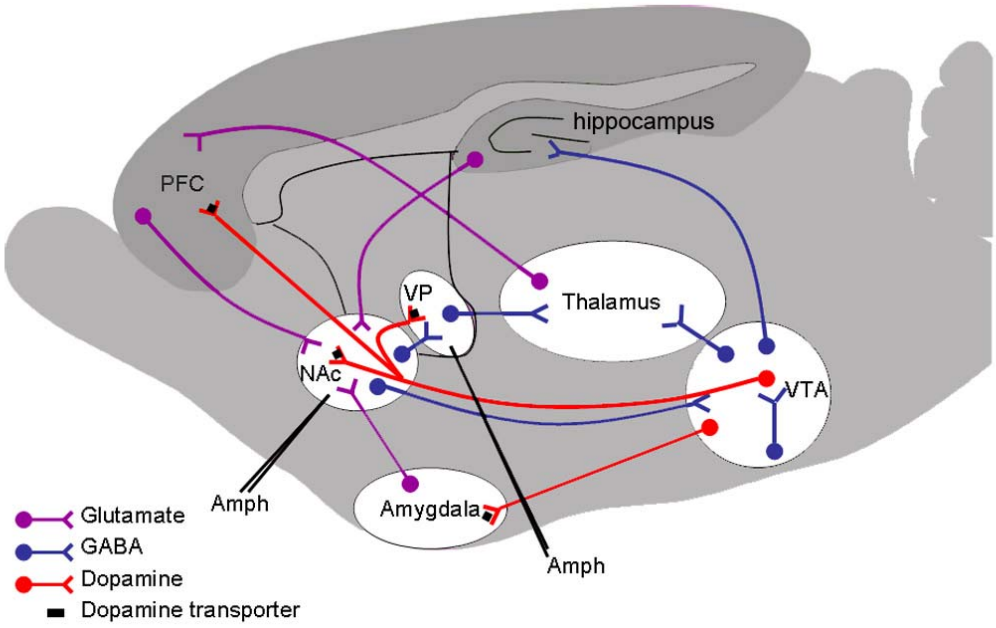

Figure 14. Cartoon depicting some of the neuronal networks involved in the brain's reward system in the mouse. The neuronal networks controlling the brain's reward system stretch over many sites, all of which have been shown to play a role in the initiation and maintenance of drug reinforcement. A major component of the pathway are dopamine neurons (depicted in red), which are located in the VTA and projecting to the NAc and other limbic structures, including the thalamus, the amygdala and the prefrontal cortex. Glutamate (purple) and GABA (gamma-aminobutyric acid) (blue) also play large roles in reward. Injections of amphetamine directly into the VP and NAc produce conditioned place preference (CPP) (Gong et al., 1996; Carr and White, 1983). Ventral tegmental area (VTA), Nucleus Accumbens (NAc) and Ventral pallidum (VP).

Individual drugs seem to vary in their targets in different parts of the limbic system. Despite this, however, the final rewarding effect is connected with an elevation of dopamine release in the NAc. Below I will describe the anatomical targets of addictive substances in mammals, before discussing the comparative anatomy of humans and fish with regard to reward in section '1.2.4 Conservation of Reward Pathways'.

The brain's reward system involves two dopaminergic pathways: the mesolimbic and mesocortical pathways (see figure 14). The mesolimbic pathway begins in the ventral tegmental area (VTA) of the midbrain and connects to the limbic system via the nucleus accumbens, the amygdala and the hippocampus, as well as to the medial prefrontal cortex. The mesocortical pathway transmits dopamine from the VTA to the frontal cortex. Both pathways are involved in motivation and emotional response and they are closely connected with each other.

Ventral tegmental area (VTA)

The ventral tegmental area (VTA) is a part of the midbrain. It consists of dopaminergic, GABAergic and glutamatergic neurons. It is part of two major dopamine pathways: the mesolimbic pathway, which projects from the VTA to the Nucleus Accumbens (NAc; see below), and the mesocortical pathway, which connects the VTA to cortical areas in the frontal lobes. Most addictive drugs produce an acute increase in the release of dopamine from the VTA neurons at their terminals in the NAc (for a review see Kauer (2003)).

Nucleus Accumbens (NAc)

The nucleus accumbens (NAc) is a collection of neurons found in the ventral striatum. 95% of neurons in the NAc are medium spiny neurons, which receive input from both the dopaminergic neurons of the VTA and the glutamatergic neurons of the hippocampus, amygdala and medial prefrontal cortex. Medium spiny neurons produce gamma-aminobutyric acid (GABA), one of the major inhibitory neurotransmitters of the central nervous system. Other neuronal types, such as spiny cholinergic interneurons, are also found.

The output neurons of the NAc send axon projections to the ventral pallidum. This projects to the mediodorsal nucleus of the dorsal thalamus, which then projects to the prefrontal cortex. Major inputs to the NAc include prefrontal association cortices, basolateral amygdala and dopaminergic neurons located in the VTA, which connect via the mesolimbic pathway.

Self stimulation studies involving electrodes implanted in the brain have identified the NAc as a major 'pleasure centre' in the brain (Olds and Milner, 1954) (See section '1.2.6 Experimental methods to study reward and drug reinforcement' for a description of Intracranial Self-Stimulation (ICSS)). In addition, amphetamine injected directly into the NAc or the VTA (see above) induces conditioned reward, as tested using a conditioned place preference paradigm) (Fletcher et al., 1998) (see '1.2.6 Experimental methods to study reward and drug reinforcement' for a detailed introduction to the conditioned place preference test).

Amygdala

The amygdala is a large nuclear mass found in the temporal lobe anterior to the hippocampus. The amygdala has a primary role in the formation and storage of memories associated with emotional events, for example fear conditioning, and also in memory modulation (the long-term storage of memories).

Hippocampus

The hippocampus is located in the medial portion of the temporal lobes. It plays major roles in both short-term memory and spatial orientation, and has more recently been implicated in drug addiction. For example, Vorel et al. (2001) show that electrical brain stimulation of the hippocampus caused reinstatement of drug-taking behaviour, in rats that had learned to lever-press for cocaine and subsequently had had this behaviour extinguished by substituting saline for cocaine. Such research implicates the hippocampus in the high rate of relapse for cocaine and amphetamine users, even after long periods of abstinence.

Prefrontal cortex

Traditionally addiction was thought to be almost entirely mediated by the limbic system. More recently, additional brain areas have been implicated - neuroimaging studies especially implicate the frontal cortex (Goldstein and Volkow, 2002). Cocaine- (Liu et al., 1998) and alcohol- (Pfefferbaum et al., 1997) dependent subjects exhibit volume losses in the frontal lobe. These decreases in volume correlate with years of use, indicating that the effect of the drug use is cumulative over time. Rodents can be trained to self-administer cocaine directly into the prefrontal cortex (Goeders and Smith, 1983).

Pedunculopontine nucleus

The pedunculopontine nucleus (PN) is a structure at the junction of the pons and the midbrain, that has been suggested to have a role in reward-related behaviours (Inglis and Winn, 1995; Winn et al., 1997). The PN strongly influences the midbrain DA neurons (Blaha et al., 1996; Blaha and Winn, 1993). Lesions of the PN impair conditioned place preference using amphetamine, (Bechara and van der Kooy, 1989; Bechara et al., 1988; Olmstead and Franklin, 1994), but not cocaine (Parker and van der Kooy, 1995).

1.2.2 The structural characteristics and molecular actions of amphetamines

Amphetamines are a widely abused class of drugs, with an estimated 35 million users worldwide (Ghodse, 2007). They are powerful and addictive stimulants. Immediate effects include euphoria, increased heart rate, blood pressure, body temperature and respiratory rate, along with increased wakefulness. Long term effects include addiction, violent behaviour, anxiety, mood disturbance, weight loss and confusion. Although some recent progress has been made, there are currently no widely-accepted pharmacological treatments for amphetamine addiction (Jayaram-Lindstrom et al., 2008). Cost-effective medicines to treat cocaine and amphetamine addiction, like methadone for heroin addiction, are much needed.

The main structural features of amphetamines are a two-carbon side chain between the phenyl ring and nitrogen, an alpha-methyl group, a primary amino group and an unsubstituted phenyl ring (see Figure 15). The two most commonly used amphetamines are amphetamine and methamphetamine. Even though they vary in structure, the two drugs show no differences in

their ability to elicit dopamine release, in their elimination rates or other pharmacokinetic properties (Melega et al., 1995).

Amphetamine increases the synaptic concentration of monoamines, such as dopamine, serotonin (5-HT) and norepinephrine, at the synaptic cleft. This is achieved through several mechanisms, as described below (see also figure 16). Amphetamine also affects the actions of other non-monoamine neurotransmitters, such as acetylcholine and glutamate.

Effects of amphetamine on dopamine transmission

Dopamine is a neurotransmitter used widely throughout the animal kingdom, in both vertebrates and invertebrates. Dopamine is synthesized in the body, first through the hydroxylation of the amino acid L-tyrosine to L-DOPA via the enzyme tyrosine hydroxylase, and then through the decarboxylation of L-DOPA by dopa decarboxylase. Dopamine is released from neurons in response to a presynaptic action potential. Dopamine is inactivated by reuptake into the neuron via the dopamine transporter (DAT), where it can then be broken down by catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO). Dopamine that is not broken down is repackaged into vesicles for reuse.

Amphetamine causes the release of dopamine from nerve terminals. It also blocks its inactivation by preventing its reuptake (Heikkila et al., 1975a; Heikkila et al., 1975b). Amphetamines are similar in structure to dopamine, so, unlike cocaine, they can enter the terminal button of the presynaptic neuron via the dopamine transporter, as well as through diffusion (Liang and Rutledge, 1982; Mack and Bonisch, 1979). In addition amphetamine frees dopamine into the nerve terminals by interacting with dopamine containing synaptic vesicles. Amphetamine also binds to monoamine oxidase in dopaminergic neurons, preventing the degradation of dopamine (Green and el Hait, 1978). Amphetamine also binds to the dopamine re-uptake transporter, causing it to act in reverse (Sulzer et al., 1995).

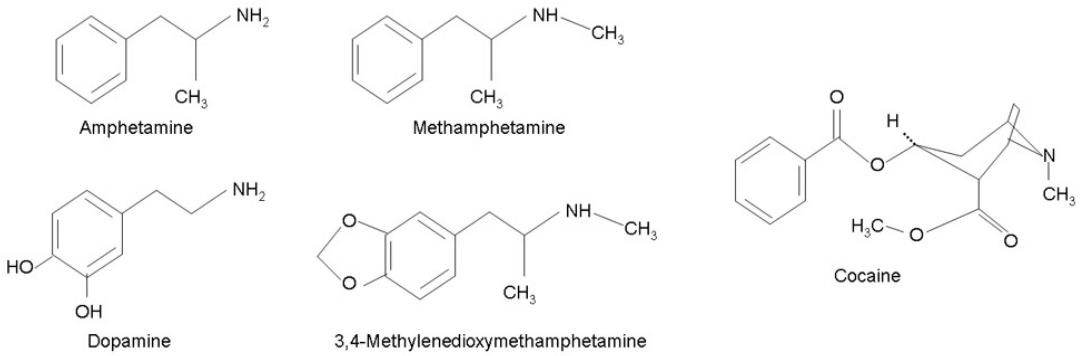

Figure 15. The chemical structure of commonly used amphetamines, compared with that of dopamine and cocaine.

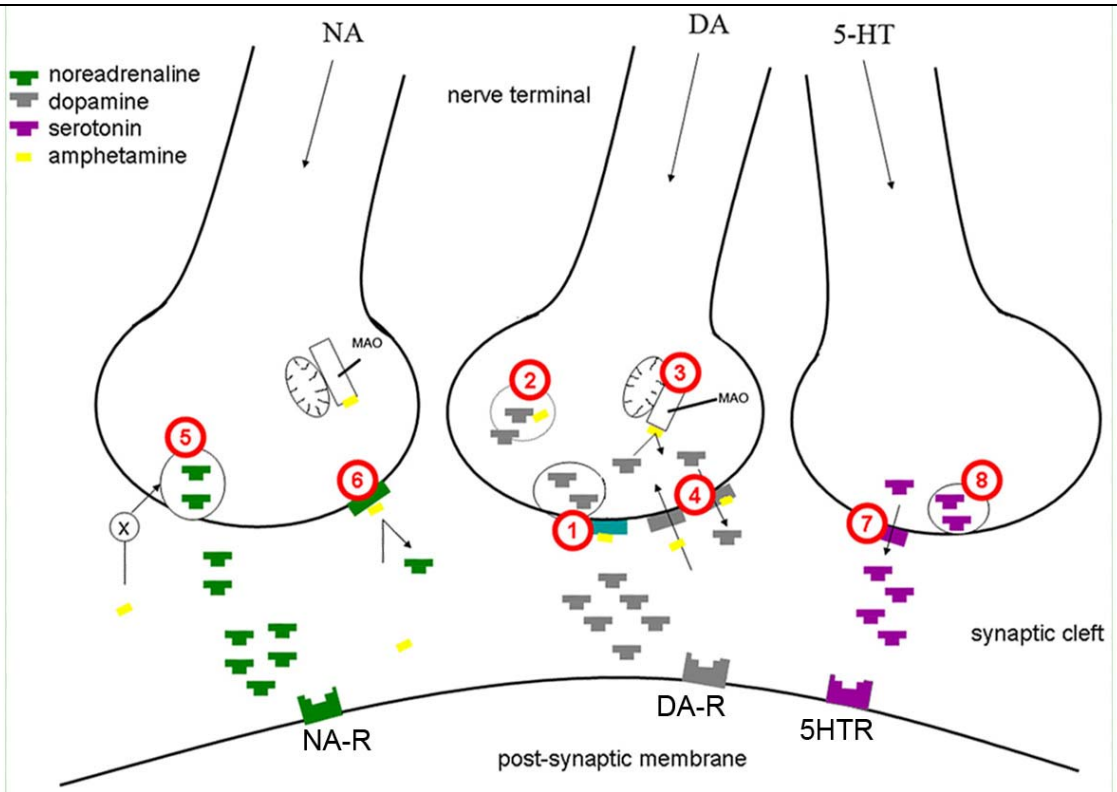

Figure 16. The molecular modes of action of amphetamine. (1) Amphetamine binds to the pre-synaptic membrane of dopaminergic neurons and induces the release of dopamine from the nerve terminal; (2) amphetamine also interacts with dopamine containing synaptic vesicles, releasing free dopamine into the nerve terminal; (3) amphetamine binds to monoamine oxidase (an enzyme bound to mitochrondria that catalyses the oxidation of monoamines) in dopaminergic neurons, preventing the degradation of dopamine and increasing the amount of dopamine in the nerve terminal; and (4) amphetamine binds to the dopamine re-uptake transporter (DAT), causing it to act in reverse and transport free dopamine out of the nerve terminal. Amphetamine has a similar effect on noradrenergic neurons; (5) it can induce the release of noradrenaline into the synaptic cleft and (6) inhibit the noradrenaline re-uptake transporter. Similar to its effect on the dopamine transporter, (7) amphetamine can also induce the serotonin transporter SERT to operate in reverse. (8) It can also increase the release of serotonin from synaptic vesicles. It is not known whether amphetamine binds SERT directly.

Effects of amphetamine on serotonin transmission

Serotonin (5-HT) is a monoamine neurotransmitter synthesized in serotonergic neurons in the central nervous system. In animals serotonin is synthesized from the amino acid L-tryptophan, by tryptophan hydroxylase and amino acid decarboxylase. The action of serotonin is terminated primarily via its reuptake from the synapse by the serotonin transporter (SERT).

Similar to its effect on the dopamine transporter, amphetamine can also induce the serotonin transporter SERT to operate in reverse (Hilber et al., 2005). This effect depends on the actions of calcium molecules, as well as on the proximity of certain transporter proteins (Hilber et al., 2005).

Serotonin also mediates the response to amphetamine indirectly. Glutamatergic afferents originating in medial prefrontal cortex regulate the firing patterns of neurons in the VTA (Gariano and Groves, 1988; Svensson and Tung, 1989). Amphetamine depresses excitatory glutamatergic synaptic transmission onto VTA neurons (Jones and Kauer, 1999). This depression is mediated through the activation of serotonin receptors – amphetamine causes the release of serotonin in the VTA, which then in turn modulates excitation of VTA neurons.

The effects of amphetamine on norepinephrine transmission

Norepinephrine (or noradrenaline) acts both as a neurotransmitter and as a hormone. It is synthesized from dopamine by dopamine β-hydroxylase. It is released from the adrenal medulla into the blood as a hormone and from neurons in the central and sympathetic nervous systems. Termination of its action occurs primarily over reuptake.

Norepinephrine levels are raised in response to amphetamine (Rothman et al., 2001). At low concentrations amphetamine can induce norepinephrine release, mediated by the norepinephrine transporter. At high concentrations amphetamine acts as a norepinephrine reuptake inhibitor, and under these circumstances norepinephrine release depends on its release from a vesicular pool (Pifl et al., 1999).

The effect of amphetamine on other non-monoamine neurotransmitters – acetylcholine and glutamate

In the central nervous system acetylcholine (ACh) coordinates motor behaviours (Mesulam, 1996), as well as more complex cognitive processes such as memory, attention and learning (Sarter et al., 2003). The limbic and the paralimbic regions of the CNS have the highest numbers of cholinergic innervations (Mesulam, 1996), and it is in these areas that ACh is thought to play a large role in addiction (Bonson et al., 2002; Childress et al., 1999; Goldstein and Volkow, 2002).

Systemically administered amphetamine causes cortical acetylcholine release. Cholinergic activity modulates dopamine transmission. There are two types of cholinergic receptors mediating the effects of ACh: nicotinic (stimulated by ACh and nicotine) and muscarinic (stimulated by ACh and muscarine). There are five distinct muscarinic receptor subtypes (M1-M5). M1 deficient mice show an increase in their response to amphetamine and injection of the nicotinic agonist cytosine can establish place preference (Gerber et al., 2001; Museo and Wise, 1994) (see section '1.2.6 Experimental methods to study reward and drug reinforcement' for a detailed description of how to establish place preference using a rewarding drug). The administration of dopamine receptor antagonists does not affect the ability of amphetamine to increase cortical acetylcholine efflux. Therefore, the release of cortical acetylcholine by amphetamine appears to take place via a complex neuronal network rather than simply increasing basal forebrain D1 or D2 receptor activity (Arnold et al., 2001).

Amphetamine also increases the extracellular levels of glutamate, the primary excitatory neurotransmitter in the brain. This effect was found in areas involved with reward: the nucleus accumbens, striatum and the prefrontal cortex (Del Arco et al., 1999). Mice lacking

the glutamate receptor 5 gene do not self-administer cocaine, and they show no locomotor response to cocaine, despite showing a cocaine-induced dopamine increase in the NAc similar to that of wildtype animals (Chiamulera et al., 2001).

1.2.3 Many neurotransmitter systems are involved concurrently in the establishment of drug reinforcement

Cocaine and amphetamine cause dopamine, serotonin and norepinephrine to accumulate in the synapses, leading to the overstimulation of the receptors for these neurotransmitters. It was traditionally though that the hedonic effects of these drugs could be attributed to the overstimulation of dopamine receptors alone. However, single knockout (KO) mice without DAT establish cocaine-conditioned place preference, as do single knockout mice for SERT (Sora et al., 1998). In addition, mice lacking the norepinephrine (NE) transporter (NET) are supersensitive to cocaine (Xu et al., 2000), indicating that neither DAT nor SERT nor NET is absolutely required for reward. However, double KO mice lacking both DAT and SERT do not exhibit cocaine-conditioned place preference (Sora et al., 2001). Surprising, extracellular dopamine levels still increase in the NAc when cocaine is administered to DAT-KO mice (Carboni et al., 2001; Mateo et al., 2004). Pharmacological inhibition of the SERT increases extracellular dopamine in the nucleus accumbens of DAT knockout mice to a similar extent as cocaine. It has been suggested that this results from adaptations in the serotonin regulation of the dopaminergic neuronal activity in the VTA of these mutant mice, adaptations which are not present in wildtype mice (Mateo et al., 2004). It is possible that neuronal adaptations occur in DAT knockout mice, to allow cocaine to increase dopamine in the nucleus accumbens, and, therefore, to respond to cocaine. Therefore, even though DAT is not necessary for cocaine-induced conditioned place preference, dopamine signaling may still be an essential mediator.

1.2.4 Conservation of Reward Pathways

The primitive nature of reward makes it possible to study drug associated reward and reinforcement in non-mammalian, and even in invertebrate, species. Dopamine has been shown to modulate responses to cocaine, nicotine and ethanol in *Drosophila* (Bainton et al., 2000). Cocaine sensitive serotonin and dopamine transporters have been identified in flies (Corey et al., 1994; Demchyshyn et al., 1994; Porzgen et al., 2001).

Many genes influencing addiction are widely conserved in function throughout the animal kingdom. For instance, the locomotor responses of flies to cocaine parallel those in mammals, indicating that dopaminergic pathways have been involved in neuronal circuits controlling movement for over one billion years (Nestler, 2000). In addition, non-mammalian model organisms have been used to identify biochemical pathways through which drugs act (for a review see Nestler, 2000). For example, normal expression of the *per* gene is required for the development of cocaine sensitization in *Drosophila* and in mouse. Flies mutant in the *Per* gene do not sensitise after repeated exposure to cocaine (Andretic et al., 1999). Further studies in mice showed that a lack of *mPer1* also prevents cocaine sensitization (Abarca et al., 2002).

The zebrafish initially became popular as a model organism because of its usefulness in developmental studies. However, it has been more recently used in behavioural studies. Although the nervous system of the fish is simpler than that in rodents, it is still able to control a variety of complex behaviours such as learning, addiction, aggression and locomotion. Behavioural paradigms have been developed to measure drug reinforcement (Gerlai et al., 2000; Lau et al., 2006; Ninkovic et al., 2006), anxiety (Gerlai et al., 2006; Ninkovic et al., 2006; Swain et al., 2004), aggression (Dlugos and Rabin, 2003; Gerlai et al., 2006), memory (Darland and Dowling, 2001; Ninkovic et al., 2006), locomotor activity (Gerlai et al., 2000; Swain et al., 2004), social preference (Dlugos and Rabin, 2003; Gerlai et al., 2000), mate choice (Turnell et al., 2003) and boldness/antipredatory behaviour (Gerlai et al., 2006; Wright et al., 2003). The investigation of the relationship between genes and complex behaviours is not straightforward (Sokolowski, 2001). A preliminary first step in such research is the identification of behavioural syndromes that can be quantified using straightforward and reliable protocols that allow high throughput screening, either with mutagenesis or naturally occurring behavioural variation. Much of the pioneering work linking genetics with behaviour has been performed using *Drosophila*. These studies have identified, among others, genes that control complex syndromes such as learning and memory, mating behaviour and circadian rhythms (Anholt and Mackay, 2004; Sokolowski, 2001). Zebrafish shares many advantages with *Drosophila*, such as its amenability to genetic screens, large number of offspring and relatively low housing costs. However, at the same time it also has the added advantage of being a vertebrate, and thus has great potential as a model for understanding the genetic basis of behavioural disorders (Guo, 2004).

Zebrafish have recently been established as a model for human drug-seeking behaviour. Adult zebrafish show a robust conditioned place preference (CPP) response to alcohol (Gerlai et al., 2000; Kily et al., 2008), opiates (Bretaud et al., 2007), cocaine (Darland and Dowling, 2001), morphine (Lau et al., 2006) and amphetamine (Ninkovic and Bally-Cuif, 2006). In the CPP paradigm one part of a two compartment area is paired with a drug stimulus, resulting in (increased) preference for that compartment (see section '1.2.6 Experimental methods to study reward and drug reinforcement' for a detailed description of the CPP test). Lau et al. (2006) developed a conditioned place preference (CPP) paradigm to show a preference for both morphine and food as rewards in larval wildtype zebrafish They also analyzed the *too few* mutant, which lack the Fez1 transcription factor leading to a selective reduction of dopamine and 5HT neurons in the basal forebrain (Levkowitz et al., 2003). They showed that this mutant failed to show a preference for morphine, while retaining its preference for food. In a separate study, Bretaud et al. (2007) show that morphine-induced CPP in zebrafish larvae can be attenuated by pretreatment with antagonists of the dopamine receptor. This indicates that the role of dopamine in reward has been evolutionary conserved in zebrafish. In a separate study the cholinergic system of the zebrafish – a system known to influence dopamine release in the NAc – was implicated in modulating the rewarding properties of amphetamine (Ninkovic et al., 2006). In this study genetic impairment of acetylcholinesterase (AChE) function in ache/+ mutants severely reduced CPP behaviour. This is paralleled in studies in mouse, showing that the pharmacological inhibition of AChE activity decreases drug reinforcement elicited by cocaine and morphine (Hikida et al., 2003).

Comparative anatomy between fish and mammals

There are some clear differences between brain of teleosts and mammals. Notably, fish have smaller cerebral hemispheres and there are differences in the layout of the forebrain (see below). In addition, a structure analogous to the nucleus accumbens (NAc) is yet to be identified and dopaminergic cells are missing from the teleost midbrain. A further complication is that the telencephalon of teleosts undergoes eversion (as opposed to invagination in other vertebrates) during embryonic development (see figure 17), making the identification of homologous structures between teleosts and other vertebrates difficult. However, recent progress has been made in the identification of structures involved in reward.

In spite of these differences, the overall organization of the zebrafish brain is similar to that of other vertebrates. Teleosts appear to have a structure homologous to the mammalian hippocampus. The mammalian hippocampus is essentially the medial edge of the cortex. The hippocampus and the cortex are formed from the pallium (dorsal telencephalon), and this is present in all vertebrates, including fish. The pallium is divided into three zones: medial, lateral and dorsal. The medial pallium (mPa) appears to be the non-mammalian, non-teleost equivalent of the hippocampus, as it facilitates spatial memory in birds and reptiles (Rodriguez et al., 2002). The eversion of the telencephalon in teleosts means that the ependymal cells of the pallium are positioned on the dorsal surface of the telencephalon and that the medial to lateral topography of the pallial areas are reversed (Rodriguez et al., 2002). As a consequence, the mPa of non-teleost vertebrates corresponds to the lateral pallium (lPa) of fish. In support of this, studies have shown that the ablation of the lPa impairs spatial memory in fish (Portavella et al., 2002; Vargas et al., 2006) (see figure 18 for the location of the lesion).

The mPa in teleosts is considered homologous to the amygdala, as it is involved in emotional conditioning. Lesions to the mPa impair the retention of a conditioned active avoidance response in goldfish (Portavella et al., 2004) (see figure 18 for the location of the lesion), as do lesions to the amygdala in mammals (Ambrogi Lorenzini et al., 1991).

The main neurotransmitter systems, such as the cholinergic, dopaminergic and noradrenergic pathways, are present in fish and they have been mapped throughout the zebrafish brain (Mueller et al., 2004; Rink and Wullimann, 2004). In mammals the mesolimbic dopaminergic system consists of dopamine neurons that have their cell bodies in the midbrain VTA and send projections to the ventral striatum, NAc, prefrontal cortex and amygdala. As mentioned above, dopaminergic neurons are absent from the midbrain of teleosts. Nevertheless, dye tracing experiments have identified a conserved ascending dopaminergic system in zebrafish and this system appears to be essential for reward responses. These neurons project from the posterior tuberculum (a structure located dorsal to the hypothalamus) to the dorsal and ventral (limbic) striatum. These projections are reminiscent of such a projection to the nucleus accumbens in mammals (Marin et al., 1995).

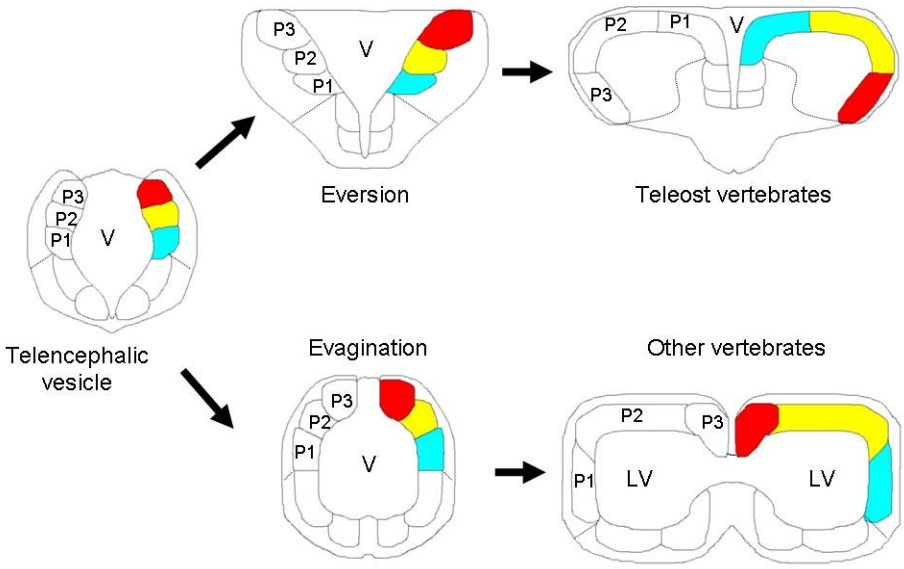

Figure 17. Schematic representation comparing the processes of embryonic development of the telencephalic vesicle in teleosts and in other vertebrates. In teleost fish the pallial region curves laterally, generating two telencephalic hemispheres with a single ventricular cavity (V) dorsally disposed upon the hemispheres. In other vertebrates the pallial wall curves inside the middle line of the telencephalic vesicle and produces two hemispheres with an internal ventricular cavity (LV) in each one. P1, P2 and P3 are the three major divisions of the pallium. Figure adapted from Portavella and Vargas (2005).

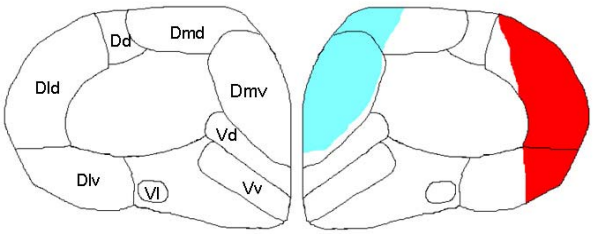

Figure 18. Graphic representation of a cross section through the anterior telencephalon of fish. The Dmv is part of the medial pallium, considered to be homologous to the amygdala of non-teleost vertebrates. The Dlv is part of the lateral pallium (lPa), considered to be homologous to the hippocampus. Coloured areas represent areas lesioned in experiments that show that the medial pallia (blue) are necessary for emotional learning and the lateral pallia (red) are necessary for spatial learning. The nomenclature used follow those reported elsewhere (Peter and Gill, 1975; Nieuwenhuys and Meek, 1990; Portavella et al., 2004a,b). Dc, area dorsalis telencephali pars centralis; Dd, area dorsalis telencephali pars dorsalis; Dld, area dorsalis telencephali pars lateralis; Dmd, area dorsalis telencephali pars medialis; Dmv, area dorsalis telencephali pars medialis ventralis; Vd, area ventralis telencephali pars dorsalis; Vl, area vetralis telencephali pars lateralis; Vv, area ventralis telencephali pars ventralis; Dlv, area dorsalis telencephali pars lateralis ventralis. Figure adapted from Portavella and Vargas (2005).

These projections, along with the high levels of D_1 dopamine receptor (Kapsimali et al., 2000) and glutamate decarboxylase expression reported in the zebrafish subpallium (Anglade et al., 1999), are features that would be expected of striatal structures when taking the situation in mammals into consideration.

Together, these studies in zebrafish as well as in other teleosts suggest that the ascending dopaminergic system of zebrafish could be homologous to the ascending dopaminergic system in mammals, even though there are differences in location in the adult midbrain vs. forebrain.

1.2.5 Mechanisms leading to addiction

Transcriptional mechanisms

Regulation of gene expression is one mechanism that should lead to changes within neurons that could even be relatively stable (Nestler et al., 1993). Even though it is likely that neural genes are regulated by hundreds of distinct types of transcription factor, two have so far been widely implicated in addiction: the cyclic-AMP response-element-binding protein (CREB) and ΔFosB.

CREB is a transcription factor involved in the regulation of many plasticity events in neurons, including long term potentiation. Overexpression of CREB in the nucleus accumbens counters the rewarding properties of opiates and cocaine; overexpression of a dominant negative CREB mutant has the reverse effect (for a review see Nestler (2000)). CREB seems to promote some aspects of addiction (for example, physical dependence), while opposing others (for example reward). CREB regulates the transcription of many genes including *c-fos*, the neurotrophin BDNF (Brain-derived neurotrophic factor), tyrosine hydroxylase, and many neuropeptides (such as somatostatin and enkephalin) (Andrisani and Dixon, 1990a; Andrisani and Dixon, 1990b; Karpinski et al., 1992; Piech-Dumas et al., 1999; Piech-Dumas and Tank, 1999; Tao et al., 1998).

ΔFosB is a member of the fos family of immediate early transcription factors and it accumulates in the nucleus accumbens after chronic exposure to several drugs of abuse (Kelz et al., 1999; Nestler et al., 1999), including opiates, cocaine, amphetamine, alcohol, nicotine and phencyclidine (PCP). Acute administration of several types of drugs of abuse rapidly raises the levels of several Fos family members, including c-Fos, FosB, Fra-1 and Fra-2, in the NAc and dorsal striatum (Hope et al., 1994). ΔFosB levels increase only slightly by acute drug exposure. However, the protein is very stable and ΔFosB isoforms accumulate with repeated drug administration. Transgenic mice in which ΔFosB is overexpressed selectively within the same set of neurons in the nucleus accumbens (an area where increased expression is seen upon drug administration) demonstrate an increased preference for cocaine (Kelz et al., 1999; Nestler et al., 1999). Induction of ΔFosB results in sensitisation. Increased levels of ΔFosB lead to increased levels of BDNF, which in turn increases the number of dendritic branches and spines on neurons in the nucleus accumbens and in the prefrontal cortex.

Relationship to neuroplasticity, including learning and memory

Repeated exposure to a drug of abuse can cause structural changes in specific neuronal cell types. For example, repeated cocaine or amphetamine exposure increases the number of dendritic branch points and spines both of medium spiny neurons in the NAc and of pyramidal neurons in the medial prefrontal cortex (Robinson and Kolb, 1997). In addition, chronic exposure to drugs can influence the birth of new neurons in the brain (see section '1.3.3.4 External factors influencing the rate of adult neurogenesis'). Some progress has been made in answering the question as to the molecular and cellular mechanisms that mediate such alterations in neural structure and neurogenesis.

Neural circuit changes underlying learning and memory depend on the strengthening or weakening of synaptic connections linked to the relevant network of neurons. Long lasting increases in strength at excitatory synapses are termed long-term potentiation (LTP); decreases in strength are classified as LTD (long-term depression) (Malenka and Bear, 2004). It has been hypothesized that drug addiction represents a potent form of learning and memory, leading to a focus on linking LTP and addiction (Kauer and Malenka, 2007). It has been demonstrated that synapses at the VTA and NAc are capable of undergoing LTP, and that LTP controls behaviours that characterise addiction (Wolf, 2003). Activation of the cAMP pathway and of CREB-mediated transcription in the hippocampus has been linked to learning, as well as to long-term potentiation (Deisseroth et al., 1995; Martin and Kandel, 1996; Silva and Murphy, 1999; Yin and Tully, 1996). Roles for neurotrophic factors and for variations in dendritic spine density have been implicated in LTP and LDT in the hippocampus (Korte et al., 1996; Luscher et al., 2000; Schuman, 1999).

1.2.6 Experimental methods to study reward and drug reinforcement

Addiction can be defined as a syndrome in which drug use prevails over other aspects of life, at the expense of social compatibility. It is obvious that addiction is a genuinely human affliction. However, aspects of addiction can be successfully modeled using animals (Sanchis-Segura and Spanagel, 2006). A number of experimental procedures are available to study the biological basis of drug addiction. The majority of these tests focus on the drugs ability to reinforce certain behaviours; that is the ability of the drug to motivate the individual to engage in behaviours leading to further drug administration.

Self-administration

One way to assess drug reinforcement is to measure the intravenous drug self-administration of laboratory animals fitted with intravenous catheters (see figure 19). The catheter is connected to a drug reservoir and the animal receives a dose of the drug by pressing a lever. The reinforcing properties of a drug are investigated by varying the effort that an animal makes in order to receive a drug dose - the more effort the animal makes, the greater the reinforcing properties of the drug (Gardner, 2000). Self-administration tests have the advantage that they measure drug-seeking and –taking, which are core features of human addiction. However, they are technically difficult to implicate and impractical for high

through-put experiments (for a review see Nestler (2000)). In addition, animals are required to perform operative responses after brain lesions or pharmacological treatments that may interfere with their ability to self-administrate.

Oral self-administration, in which drugs are self-administered through the drinking water, is another method achieved in rodents (Crabbe et al., 1990; De Waele et al., 1992). Mice are presented with two bottles in their home cage: one containing water and another containing a solution of water and drug. This has been achieved in rodents for many drugs, including amphetamine (Meliska et al., 1995; Ufer et al., 1999). Oral self-administration has the advantage of being technically less demanding. Possible disadvantages of oral self-administration are the stability of the drug in the water at room temperature, the nutritional (caloric) value of the drug and the taste of the drug.

Intracranial self-stimulation (ICSS)

In self stimulation studies, electrodes are positioned into specific brain regions, which animals can then stimulate electrically (Olds and Milner, 1954) (see figure 20). Olds and Olds (1963) found that stimulation of a large range of limbic and diencephalic structures is rewarding – numerous further investigations have found many more. Of the various reward sites identified, electrodes positioned in the ventral tegmental area (VTA) or DA projections (mesostriatal pathway) to the NAc produce the most reliable intracranial self-stimulation (ICSS) response rate (Wise, 1996).

The powerful nature of ICSS is demonstrated by the readiness of animals to learn and execute stimulation-producing behaviour (for a review see Shippenberg and Koob (2002)). Drugs of abuse can decrease thresholds for ICSS, and there is good correspondence between the ability of a drug to decrease the threshold of ICSS and its addictive potential (Kornetsky et al., 1979).

Conditioned place preference

The conditioned place preference (CPP) paradigm provides a way of estimating the subjective effects of drugs (Bardo et al., 1995) (see figure 21). The apparatus used in CPP experiments has two compartments distinguishable from each other by environmental cues, such as differences in lighting or colour. Administration of a drug is paired with one environment; administration of a saline control is paired with another. This procedure is repeated for several days, during which the animal develops an association between the subjective effects of the drug and the environmental cues where the drug is administered. When later given a choice, the animals spend more time in the environment in which they were previously under the influence of a reinforcing drug. This learned association between the environmental cues and the effect of the drug is the basis for the CPP test.

In an unbiased design the environments are designed so that the animal can discriminate between the two, but does not exhibit a preference for either side prior to conditioning. In a biased design, animals exhibit preference for one side or another prior to conditioning. In the case where the drug is thought to have positive reinforcing effects, the drug is paired with the least preferred side. Disadvantages of a biased design include that the initial place preference

has to be accurately determined for each experimental run (for unbiased design, after the initial tests to control for its unbiased nature, it is not usual to establish the initial place preference, although this can be advantageous (see below)). Secondly, a biased design may produce false positives, if the drug being tested has a strong anxiolytic effect, which could overcome the initial aversion to the non-preferred compartment (Tzschentke, 1998). However, with appropriate controls a biased design can be used successfully (Ninkovic and Bally-Cuif, 2006). An added advantage of determining the initial place preference is that the investigator can ascertain that the animal sees and responds correctly to the visual cues of the system.

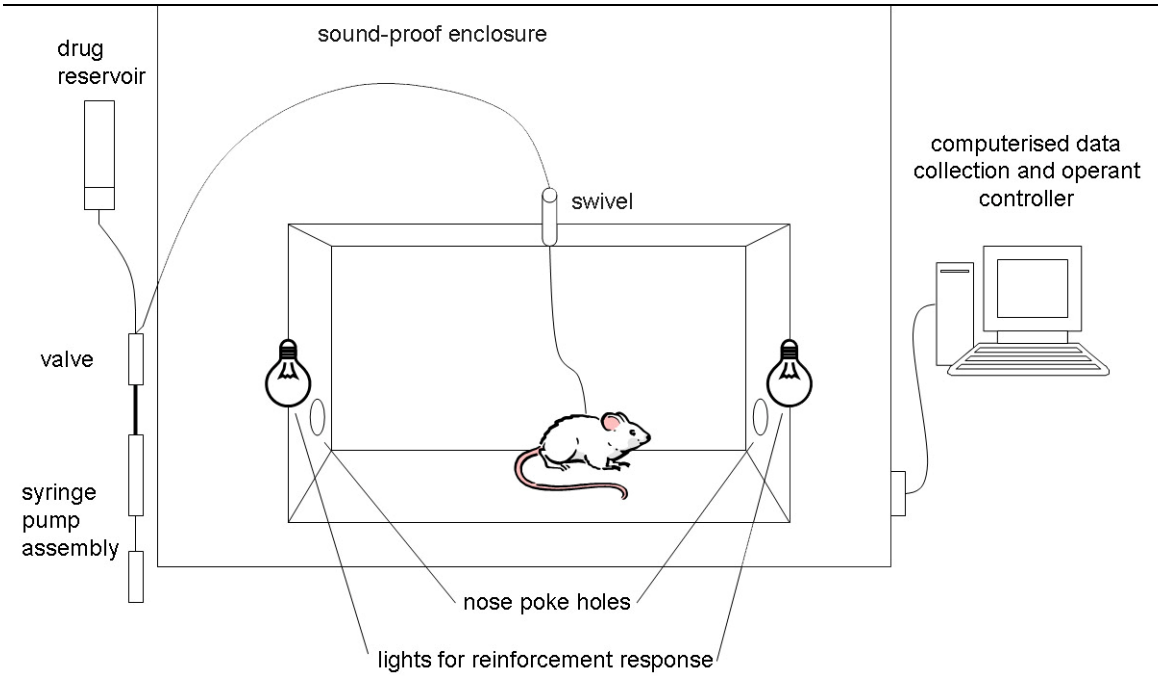

Figure 19. Operant system for intravenous self administration of drugs in mice (The mouse pokes its nose through a hole in order to receive a dose of the drug directly into a vein. At the same time a light is turned on (or a tone is played), in order to reinforce the response. A nose poke hole not connected to a drug source acts as a control. Adapted from Crawley (2000).

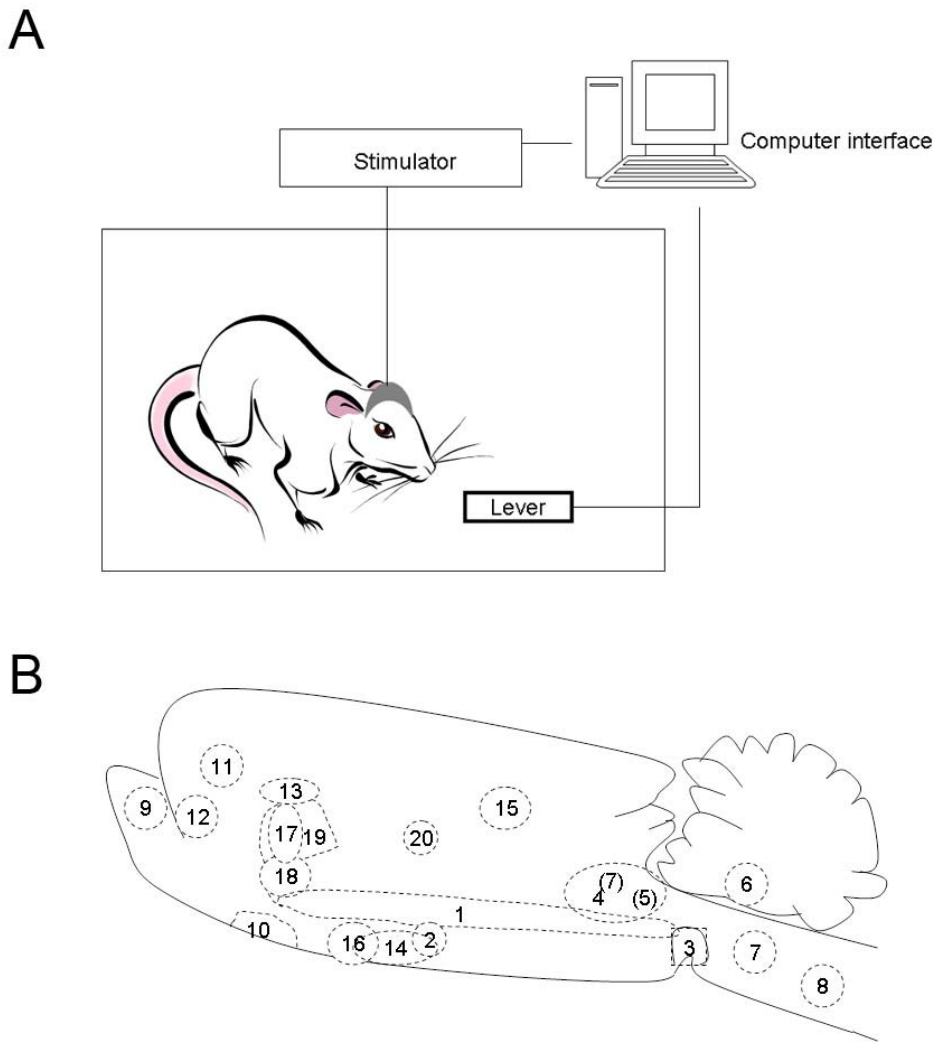

Figure 20. Animals will perform arbitrary operant responses in order to obtain electrical stimulation of some brain regions. A. Simulating electrodes are implanted in to the brain (in this case depicted in the rat). Animals are trained to press levers in order to receive brief pulses of electrical stimulation in the chosen brain areas. B. Areas of the rat brain where brain stimulation has been shown to be rewarding: 1. medial forebrain bundle sites, including the anterior, posterior and lateral hypothalamus; 2. ventromedial hypothalamus; 3. substantia nigra and ventral tegmental area (VTA); 4. midline mesencephalon, including the regions of the dorsal and medial raphe nuclei; 5. region of locus coeruleus; 6. deep cerebellar nuclei and decussation of the brachium conjunctivum; 7. regions of the mesencephalic and motor nuclei of the trigeminal nerve; 8. nucleus of the solitary tract; 9. olfactory bulb; 10. olfactory tubercle; 11. medial frontal cortex; 12. sulcal frontal cortex; 13. anterior cingulate cortex; 14. entorhinal cortex; 15. hippocampus; 16. amygdala; 17. medial and lateral septal regions; 18. nucleus accumbens; 19. caudate nucleus; and 20. dorso-medial thalamus. Adapted from Wise (1996).

An advantage of the CPP test is that it is much easier to conduct that intracranial self-stimulation or intravenal self-administration studies – it does not require special equipment or the surgical preparation of animals. Therefore, a comparatively large number of animals can be tested within a reasonably short time period. A second advantage of CPP is that the animals are tested in a drug-free state (in our case, the next day, when the drug is no longer present in the brain). This excludes problems with self-administration occurring under the influence of the drug, due to drug induced motor effects, aversion or toxicity. Although the CPP method does not directly measure drug reinforcement, the concordance between CPP and self-administration studies is good.

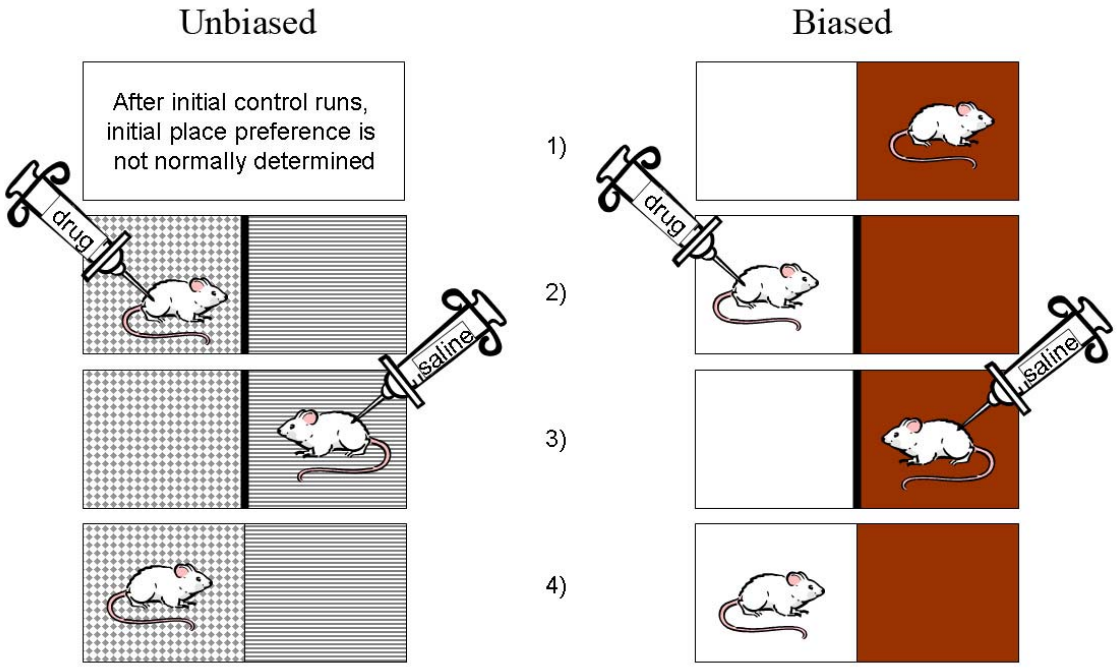

Figure 21. Conditioned place preference set-up, illustrating the differences between biased and un-biased tests. For the biased test, initial place preference is determined and the drug is paired with the least preferred side, while the vehicle is paired with the preferred side. In the case of the unbiased test, initial tests have shown that the animal does not have an initial place preference. The drug is then randomly paired with one compartment; the vehicle with the other. In both cases, if the drug has reinforcing properties, the animal will change its preference towards the side paired with the drug.

Locomotor activity

Another method of assessing an animal's response to a drug is to measure locomotor activity – acute administration of most drugs of abuse increases locomotor activity (Wolf, 1998). Animal studies have linked increased dopamine activity in the NAc and increased locomotion to the behavioural response of drug-seeking following exposure to cocaine, amphetamine, PCP and nicotine. However, this method does not measure reward directly, and the relationship between locomotor responses and drug reward are a matter of some debate (Nestler, 2000).

Due to technical difficulties involving the manipulation of the brain and the fact that zebrafish are swimming in water, we can not at this time perform self-administration studies in zebrafish. In addition, zebrafish do not show a locomotor response to amphetamine (J. Ninkowic, unpublished study). However, previous work in my lab has established a reliable zebrafish CPP model for amphetamine (Ninkovic and Bally-Cuif, 2006). This was used to isolate the mutant *no addiction* (*nad*^{dne3256}), which I have further analysed in this work.

1.2.7 Identification of genes involved in addiction

A predisposition to drug addiction is determined by complex genetic and environmental factors (for a review see Goldman et al. (2005)). The genetic component is likely to be multigenic and heterogenous – most individuals who sample drugs of abuse do not progress to addiction. Nevertheless, approximately 40-60% of the risk for alcohol, cocaine or opiate addiction appears to be inherited (Lin et al., 1996; Nestler, 2000; Pickens et al., 1991; Tsuang et al., 1996). Therefore, large-scale genomic approaches are vital to understanding the complex mechanisms underlying addiction.

QTL

A commonly used approach is the inbreeding of selected mice, which display different degrees of addiction-related behaviours, in order to then do an association study correlating a particular behaviour with particular genetic polymorphisms (Crabbe et al., 1999). This has produced limited results, due to the time and effort involved in generating these mice and that these investigators are mostly dealing with quantitative traits. Firstly a chromosomal region is linked to a certain behaviour. This region is then termed a quantitative trait loci (QTL). A broad region of the chromosome is slowly narrowed down, so that eventually only one gene, then termed a quantitative trait gene (QTG) remains. To date, only one QTG has been identified in addiction research – Mpdz, which encodes the "multiple PDZ domain protein", affects the severity of acute alcohol and pentobarbital withdrawal (Shirley et al., 2004). This protein normally facilitates the formation of large protein scaffold assemblies found at tight junctions (Ullmer et al., 1998).

Candidate gene approach

Transgenic animals, where the function of a gene is perturbed or a gene product is overexpressed, have been successfully used in elucidating the mechanisms of addiction. For example, mice lacking the serotonin 5HT1B receptor self-administer cocaine and alcohol at higher levels than wild-type controls, and in addition these mice also express higher levels of ΔFosB under basal conditions. See also section '1.2.3 Many neurotransmitter systems are involved concurrently in the establishment of drug reinforcement' for more examples of the use of the candidate gene approach to elucidate the mechanisms of reward. One problem with this approach is that the genetic changes can be compensated for during development (although this problem is more recently being addressed with inducible transgenic lines). Even though the candidate gene approach has been successfully used to confirm and extend our knowledge of addiction, it is hampered by our limited knowledge about the nature of addiction in humans.

Forward genetic approaches

Forward genetic approaches, which do not rely on the prior selection of a candidate, are useful for recovering new genes involved in many biological processes. Such screens were first carried out in *Drosophila* and they have since been successfully carried out in zebrafish (Mullins et al., 1994; Solnica-Krezel et al., 1994) and mouse (for a review see Kile and Hilton (2005)). Genetic mutations are enduced either through chemical mutagenesis (e.g. the methylating agent ethylnitrosourea (ENU)), or more recently through insertional mutagens (e.g. retroviruses) (Gaiano et al., 1996) (see also figure 22). Zebrafish screens related to drug abuse have been carried out for amphetamine (Ninkovic and Bally-Cuif, 2006) and cocaine (Darland and Dowling, 2001).

Microarray studies

It is now widely accepted that the brain is remodelled during the course of development of drug dependence and that changes in transcriptional regulation triggered by drug use play a central role in this remodelling (Yuferov et al., 2005). In experimental models repeated administration of a drug leads to an excess of or diminished production of multiple transcribed genes (Bahi and Dreyer, 2005; Funada et al., 2004). Even a single injection of morphine, for example, can lead to gene expression changes (Loguinov et al., 2001).

Functional genomics studies use microarray analysis, in which thousands of genes (or fragments of them) are arrayed on a chip. The advantage of this approach is the ability to discover the role of genes that were previously unsuspected. A consequence of this approach is the vast amount of data that is produced, which needs to be analysed (see below for more discussions on bioinformatic techniques).

One of the aims of my thesis is the molecular characterization of *no addiction* ($nad^{dne3256}$), a dominant mutation that causes a failure to respond to amphetamine in a conditioned place preference (CPP) test. This mutation was isolated in an ENU screen. As a result, it is impossible in advance to know which molecular pathways are affected. Therefore, it was

decided to implement a microarray approach, as this does not depend on prior knowledge of the molecular deficiencies in these mutants.

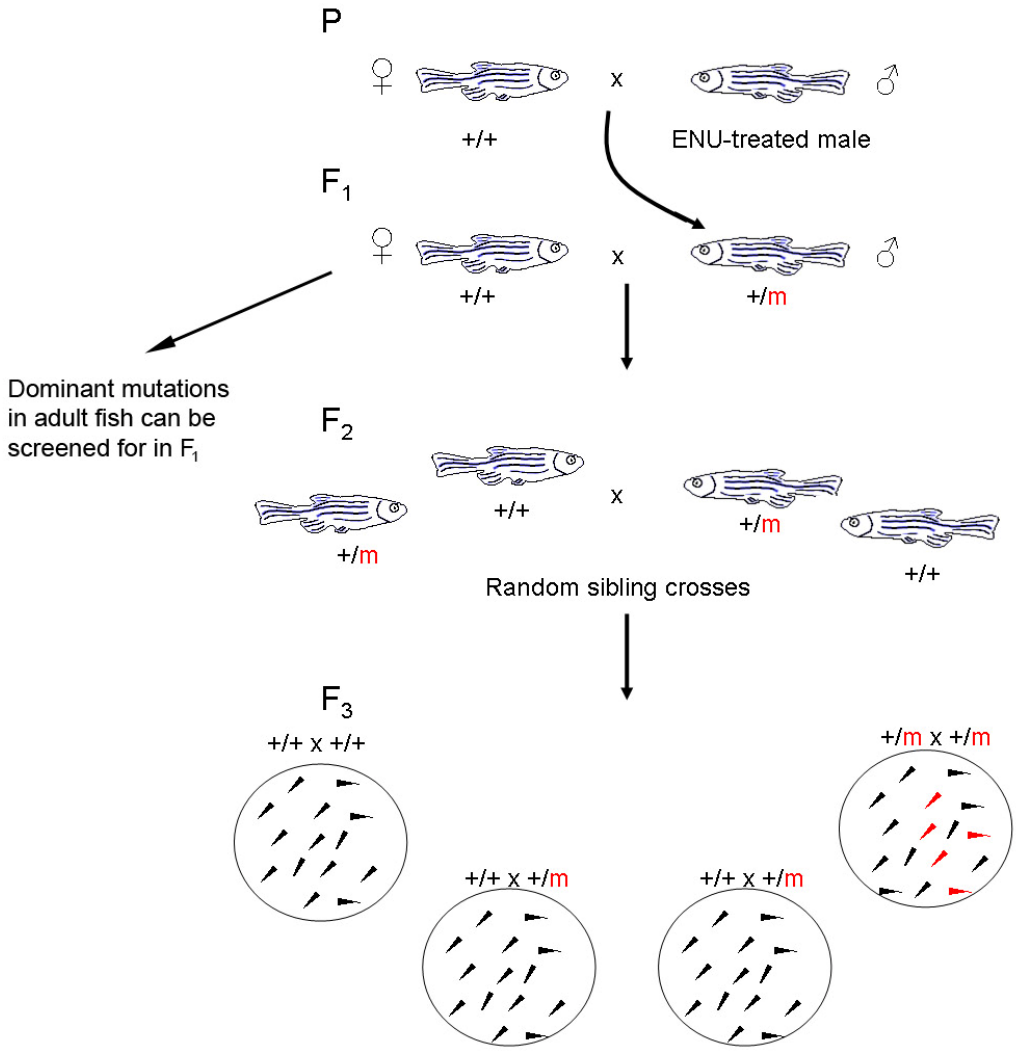

Figure 22. Mutagenesis screen in zebrafish. In F_2 screens, such as the large scale ones in Tübingen and Boston, males are first treated with a mutagen, such as ethylnitrosourea (ENU), in order to create hundreds of point mutations in the male spermatogonia. ENU-treated males are then crossed to wildtype females, in order to produce the F_1 heterozygous progeny. F_1 fish are then inbred to siblings, to create F_2 families, of which 50% carry the genetic mutation. Siblings from the F_2 families are crossed with each other, leading to a F_3 progeny, which are 25% wildtype (+/+), 50% heterozygous (+/m) and 25% homozygous (m/m) for a specific mutation. When screening for dominant mutations (that is mutations that can be detected in the heterozygotes), the F_1 generation can be screened already.

1.2.8 Bioinformatics approaches for the analysis of large data-sets relating to addiction

Gene Ontology

The Gene Ontology (GO) project classifies genes into a hierarchy, grouping gene products with similar functions together (Harris et al., 2004). Because GO is hierarchal, every time a gene is assigned to a category, it will, in addition, be assigned to all of the parent categories of this term. Once the genes have been categorised, a next step can be to see if there are any pathways or classes that are significantly upregulated in the same group verses, for example, the whole database. This involves the comparison of a list of differentially regulated genes to that of a specific pathway or classification to identify if there are more matches than would be expected by chance in a process celled GO enrichment analysis (Draghici et al., 2003).

Network analysis

Network analysis links genes based on functional data. One large scale project – the Karg (Knowledgebase for Addiction Related Genes) database – uses information from the literature in order to produce a comprehensive database of genes regulated through drug use (Li et al., 2008). The Karg database identifies 1500 human genes linked to addiction. Five molecular pathways respond to four different drugs of addiction – cocaine, alcohol, opiates and nicotine. These are neuroactive ligand-receptor interaction, long-term potentiation, GnRH signalling pathway, MAPK signalling pathway and Gap junctions.

A major draw-back for all of these methods is the large number of genes for which no functional annotation exists – these genes are effectively excluded from the analysis (Curtis et al., 2005). This draw back is particularly evident when using networks to analysis zebrafish microarrays. There is comparatively less literature on zebrafish genetics than on mouse genetics – as a result, the analysis of our microarray data using established methods failed to link any of the genes in a meaningful way. As a result, we developed the ZFISHDB database (publically available under http://mips.helmholtz-muenchen.de/zfishdb/) in collaboration with the group "computational modelling in biology" at the Helmholtz Zentrum München. This database links zebrafish genes with their mouse orthologues and integrates information on mouse genes into the network analysis.

1.3. Adult neurogenesis

Adult neurogenesis - the process in which neural progenitor cells (NPCs) develop into neurons, which are then integrated into existing circuits in the brain - is a conserved feature throughout the animal kingdom, having been described in many different species, including moths, crickets, fish, birds, rodents, non-human primates and humans (Cayre et al., 2007; Dufour and Gadenne, 2006; Gould et al., 1999b; Gould et al., 1999c; Gross, 2000; Zupanc, 2006). In mammals relatively high rates of neurogenesis are found in the subependymal zone (SEZ) of the lateral wall of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Neurons born in the adult SEZ migrate over a great distance through the rostral migratory stream and they form granule neurons and periglomerular neurons in the olfactory bulb. Neurons born in the SGZ migrate into the granule cell layer of the dentate gyrus and become dentate granular cells. These newborn neurons integrate into the existing circuitry and receive functional input (Zhao et al., 2008). Whether neurogenesis occurs in areas of the adult mammalian brain other than at the SEZ and SGZ remains controversial (reviewed in Gould (2007)).

The new neurons are generated from a population of dividing cells known as neural stem/progenitor cells. There is some controversy over what separates stem cells and progenitor cells, and some authors use the names interchangeably. Stem cells are defined as having the potential to renew indefinitely, whereas progenitors can divide only a finite number of times. Stem cells are characterised by the ability to renew themselves through mitotic cell division and the ability to differentiate into a various specialized cell types. Progenitors also have the ability to generate different cell types, but they are said to be in a further stage of cell differentiation – thus they are in the 'middle' between stem cells and fully differentiated cells. The characteristics of the NPCs of the two major mammalian populations – SGZ and SEZ – differ from each other (figure 23).

In the hippocampus there are type 1 cells, which are putative stem cells with perhaps an unlimited capacity for self renewal. Type 1 hippocampal progenitors have long processes and express nestin, glial fibrillary acidic protein (GFAP) and the Sry-related HMG box transcription factor, Sox2 (Fukuda et al., 2003; Garcia et al., 2004; Suh et al., 2007). Although they express the astrocyte maker GFAP, they are morphologically and functionally different from mature astrocytes. Type 1 cells generate type 2a cells, which then go on to generate type 2b cells. Type 2 cells only have short processes and they do not express GFAP. It has been shown that type 2 cells have a limited ability to self-renew and that a single Sox2-positive cell can give rise to a neuron and an astrocyte (Suh et al., 2007). It is widely thought that type 2 cells might arise from type 1 cells, although evidence for this is lacking (Zhao et al., 2008). Type 2 cells are thought to generate type 3 cells, which then differentiate into neurons.

The SEZ-ependymal region contains at least four different cell types – A, B, C and E – defined by their morphology, ultrastructure and molecular markers (Doetsch et al., 1997). Type A cells are young, migrating neurons, which form chains ensheathed by B cells, which are astrocytes. Type C cells are highly proliferative precursors, which form clusters next to the chains of migrating type A cells. The SEZ is separated from the ventricle cavity by a layer of ependymal cells (type E cells). Several lines of evidence support the hypothesis that type B cells generate type C cell, which then generate type A cells. Type A cells do divide (Lois and Alvarez-Buylla, 1994), so it is possible that they simply generate more type A cells.

However, purified type A cells in culture do not appear to be self renewing (Lim and Alvarez-Buylla, 1999). In contrast, isolated type B and type C cells give rise to large colonies of type A cells. In addition, if you pharmacologically ablate type C and type A cells with antimitotic drugs, SVZ type B cells generate new type C cells (Doetsch et al., 1999) and type B cells labelled with a retrovirus also resulted in the generation of labelled neurons that migrated and integrates in the olfactory bulb. Thus, it appears that type B cells function as the primary precursors for new neurons.

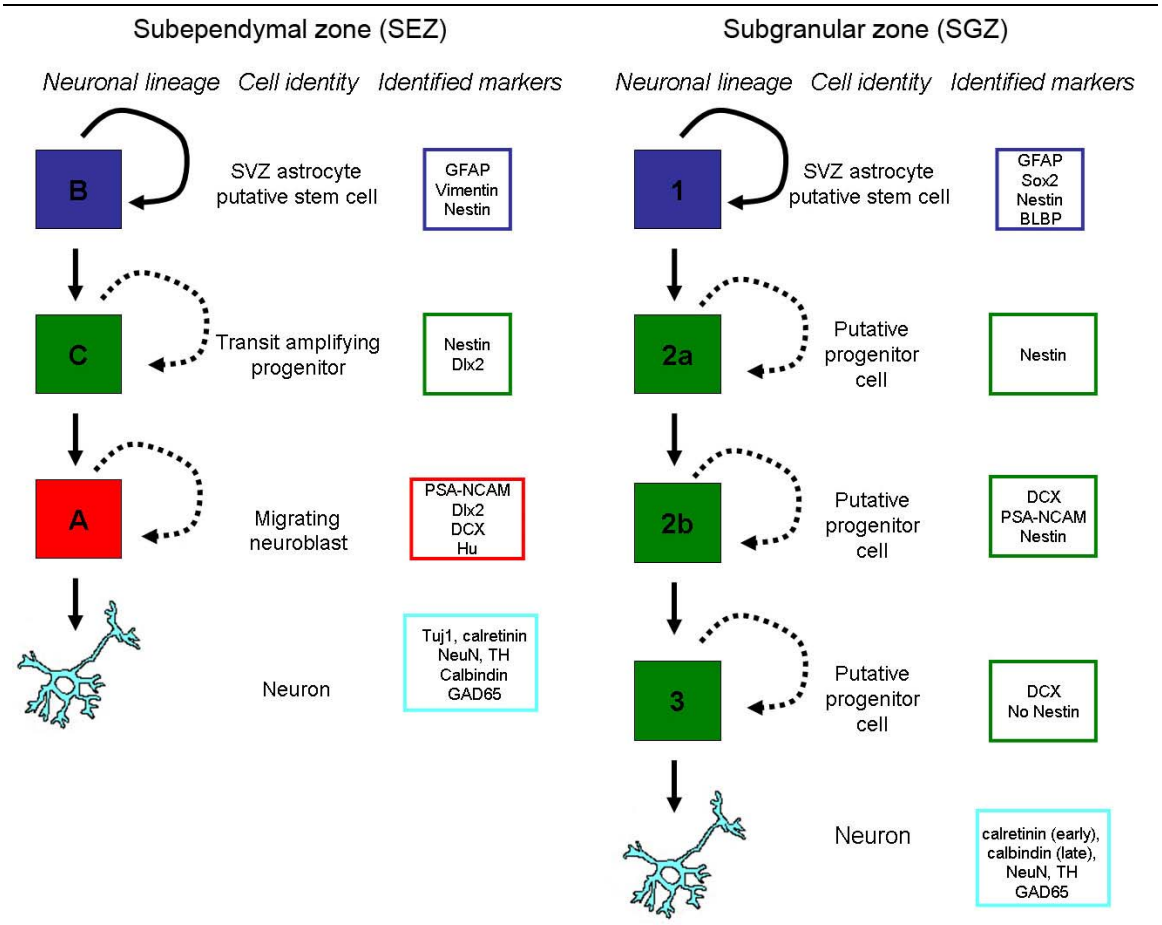

Figure 23. Adult neurogenesis is the process in which neural progenitor cells (NPCs) develop into neurons, which are then integrated into existing circuits in the brain. A) Proposed sequence of cell types in adult neurogenesis of the subventricular zone. NPCs (or 'B' cells) in the subventricular zone (SEZ) generate 'C' cells, which then generate 'A' cells. These 'A' cells migrate, before forming neurons. There are several markers to identify the individual cell types at each stage; in case of the neuronal markers, some will only detect specific types of neurons. B) Proposed sequence of cell types in adult neurogenesis in the hippocampus. Type 1 cells are the equivalent of the 'B' cells in the subgranular zone of the hippocampus. It is though that these type 1 cells generate type 2a cells, followed by type 2b cells and type 3 cells, before forming neurons. Although many of the markers are the same as those used in SVZ adult neurogenesis, as this figure illustrates, there are some important differences. Adapted from (Abrous et al., 2005; Doetsch et al., 1997; Doetsch et al., 1999; Zhao et al., 2006).

1.3.1 Zebrafish as a model to study adult neurogenesis

In fish, adult neurogenesis occurs in many more places and at a greater magnitude than in mammals (see figure 24). For one particular teleost species, *Apteronotus leptorhynchus*, it has been estimated that 0.2% its brain's cells are proliferating at any one time (Zupanc and Horschke, 1995). Although the brain of the fish grows through-out its life time, several lines of evidence have established adult neurogenesis in the fish as a separate phenomenon, distinct from general growth. Firstly, adult neurogenesis in the fish occurs in niches, mostly in ventricular zones (Ekstrom et al., 2001; Zupanc et al., 2005). Secondly, as detailed below, the generation of olfactory bulb (OB) neurons in the zebrafish resembles that of olfactory bulb adult neurogenesis in mammals (Adolf et al., 2006). Proliferating cells have been shown to leave the ventral subpallial ventricle and then enter the olfactory bulb, and a stripe of PSA-NCAM immunoreactive cells reaching into the OB is the likely migration route of the newborn neurons. The newborn neurons also resemble molecularly those in the mouse. For example, they express the transcription factors *pax6b* and *sox2* (Adolf et al., 2006; Chapouton et al., 2006). In addition *gad67*+/TH− neurons are added to the internal layer (GABAergic interneurons) and *gad67*+/TH+ (TH-positive interneurons) to the outer part of the zebrafish OB (Adolf et al., 2006). Unlike mammals, astrocytes have not been identified in the adult zebrafish brain. However they do contain radial glia cells and these cells exhibit proliferative activity, suggesting that they give rise to newborn neurons (Adolf et al., 2006; Grandel et al., 2006; Lam et al., 2009).

In zebrafish, experiments involving cumulative BrdU (a DNA synthesis marker labelling dividing cells), followed by long term tracing and subsequent staining with the proliferation marker PCNA, show that the proliferation zones of the adult brain contain both fast- and slow-dividing precursors. These slow-dividing precursors do not dilute the BrdU label, remaining in cycle over long periods and are thus considered to be adult NPCs (Adolf et al., 2006; Chapouton et al., 2006).

As the zebrafish maintains many niches of adult neurogenesis and these have the capacity to generate many more different neural subtypes than mouse, it is a good model to study adult neurogenesis. In this thesis I use the zebrafish in order to study the affects of amphetamine on proliferation and differentiation.

1.3.2 The role of adult neurogenesis

Neuroplasticity is the ability of the brain to reorganise neural pathways, based on new experiences encountered throughout life. Traditionally it was thought that the number of neurons in the brain was fixed very early on in life and that neuroplasticity was achieved through the strengthening of synapses, due to alterations in neurotransmitter receptors, numbers of synapses, structure of synapses, and transmitter release mechanisms. However, recent research has shown that adult neurogenesis also plays an important role in adult brain plasticity. Firstly, of the thousands of new neurons formed daily, most of them are integrated into functional circuits (Cameron and McKay, 2001). Secondly, adult neurogenesis is a highly conserved mechanism occurring across the animal kingdom from insects to humans (see above for references). Thirdly, environmental changes, genetics and drugs all have the ability

to alter neurogenesis in a manner that is consistent with newborn neurons having an impact on plastic processes such as learning and mood (Duman et al., 2001a; Gould et al., 1999a).

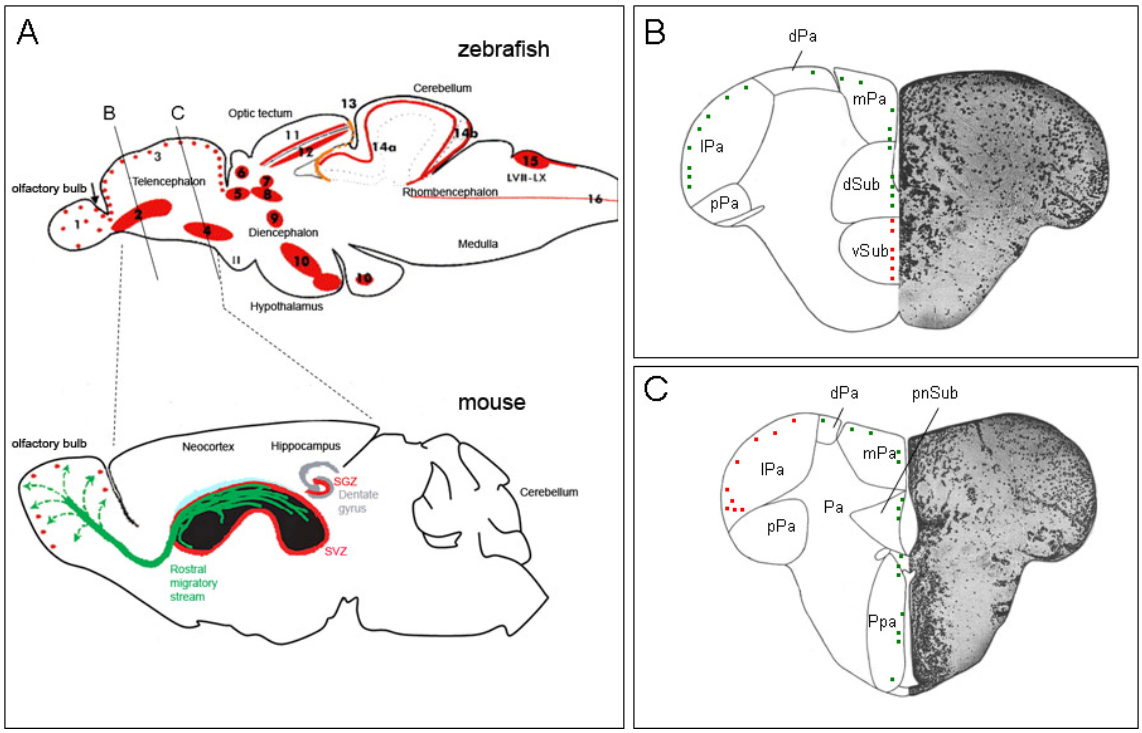

Figure 24. A comparison of adult neurogenesis in fish and mammals. A) Schematic, saggital views of zebrafish and mouse brains, showing areas of adult neurogenesis in red. Not drawn to scale. Adult neurogenesis occurs in many places in the brain of the fish, and it is largely restricted to the ventricular areas. For the zebrafish brain, areas of adult neurogenesis are as follows: Olfactory bulb: (1) scattered proliferation in the olfactory bulb; telencephalic proliferation zones: (2) ventral and (3) dorsal proliferation zones; Diencephalic proliferation zones: (4) preoptic, (5) ventral thalamic, (6) habenular, (7) pretectal, (8) dorsal thalamic, (9) posterior tubercular and (10) hypothalamic proliferation zones; Mesencephalic proliferation zones: (11) tectal and (12) torus longitudinalis proliferation zones. (13) Posterior mesencephalic lamina connects the tectum to the cerebellum. It starts dorsally at the proliferative tectal margin, continues as nonproliferative lamina and becomes proliferative again as it touches the cerebellar surface. Cerebellar proliferation zones: (14a) molecular layer proliferation zone extending through the valvula and copus cerebelli. (14b) Proliferation zone of the cerebellar caudal lobe extending from the ventricular lumen through the granular layer to its surface; Proliferation zones in the medulla oblongata: (15) proliferation zones in the facial (LVII) and vagal (LX) lobes extending caudally into the nucleus of Cajal. (16) Rhombencephalic ventricular proliferation zone extends into the spinal cord. The arrow points to an accumulation of proliferating cells at the junction of the olfactory bulb with the dorsal telencephalon (figure after Grandel et al., (2006). In contrast, in mammals only two major sites of adult neurogenesis have been identified – the subependymal zone (SVZ) of the lateral wall of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. B. Cross section of the zebrafish telencephalon (at the level indicated on A, as 'B'). Labelling studies with BrdU and PSA-NCAM indicate that proliferating cells from the ventral subpallium (orange dots) migrate to the olfactory bulb. Green dots represent the

remaining proliferating cells. C) Cross section of the zebrafish telencephalon (at the level indicated in A, as 'C'). Proliferating cells in the lateral and posterior pallium - an area thought to be the equivalent of the mammalian hippocampus - are represented schematically in red. The other proliferating cells are represented in green. Figure A adapted from Grandel et al. (2006) and from Zhao et al. (2008). Figure B and C adapted from Wulimann et al. (1996). Abbreviations: dPa: dorsal pallium, dSub: dorsal subpallium, lPa: lateral pallium, mPa: medial pallium, pnSub: postcommisural nucleus of the ventral subpallium, Ppa: preoptic nucleus, pPa: posterior pallium, TelV: telencephalic ventricle, vSub: ventral subpallium.

Hippocampus

The role of adult neurogenesis in the hippocampus remains controversial. It appears that the main function of the hippocampus itself is not long-term memory storage, but rather an area where memories at prepared for long-term storage in the cortex. However, evidence also suggests that the hippocampus plays a direct role in the storing and processing of spatial information. For example, hippocampal lesions impair navigation in rats (Morris et al., 1982) and London taxi drivers have larger hippocampi than the general population (Maguire et al., 2000). Learning enhances adult neurogenesis in the hippocampus (Gould et al., 1999a). An enriched environment or running have also been shown to increase neurogenesis and at the same time improve spatial memory, although a causal link remains to be established (Nilsson et al., 1999; van Praag et al., 1999a).

Olfactory bulb

Thousands of newly born type A neurons migrate into the OB every day (Lois and Alvarez-Buylla, 1994). However, only a fraction of these cells survive to complete their differentiation. The function of these newly born neurons is not yet clear. It is possible that they optimise olfactory circuitry, in that the new neurons are integrated into olfactory circuits that are already responding to environmental signals. Or, it has also been postulated that the continuous replacement of neurons in the OB allows for adjustment of olfactory circuitry as the environment or relevance of odours change (Alvarez-Buylla and Garcia-Verdugo, 2002). In the olfactory bulb, the reduction of new interneuron integration in neural cell adhesion molecule-deficient mice is correlated with deficits in odour discrimination (Gheusi et al., 2000). Odour enrichment results in improved survival of progenitors, as well as improved odour discrimination and odour memory (Rochefort et al., 2002). The effect on odour enrichment on progenitor survival is specific, as enriched odour exposure did not influence hippocampal neurogenesis.

1.3.3 Mechanisms regulating adult neural progenitor cells

Neural stem/progenitor cells are thought to reside in niches – zones in which NPCs are retained after embryonic development for the production of new cells of the nervous system (for a review see Conover and Notti (2008)). The NPCs are connected with their somatic cell neighbours. This connection is important to keep the cells within their niche and also for the maintenance of the germinal characteristics of the niche in the environment of adult tissue (for

a review see Fuchs et al. (2004)). The proliferation and differentiation of NPCs at the niche is influenced by a complex array of diffusible signalling molecules, including neurotransmitters and growth factors, as well as intrinsic factors.

1.3.3.1 Neurotransmitters

The neural stem cell niche is regulated by a variety to neurotransmitters, including the catecholamines noradrenaline and dopamine, as well as serotonin, gamma-aminobutyric acid (GABA) and nitric oxide (NO).

Noradrenaline

Noradrenaline supports proliferation in the SGZ of the dentate gyrus. Noradrenergic fibres closely associate with both proliferating progenitors and immature migrating neurons (Rizk et al., 2006). The pharmacological-mediated increase of noradrenaline release or the inhibition of noradrenaline reuptake increases proliferation in the dentate gyrus (Malberg et al., 2000; Rizk et al., 2006). Conversely, pharmacological ablation of noradrenergic neurons decreases SZG proliferation. Noradrenaline also promotes the survival of newborn hippocampal neurons – an effect believed to be mediated through increases in brain-derived neurotrophic factor (BDNF) expression (Rizk et al., 2006).

Dopamine

Noradrenergic fibers have not been found in the SVZ, however, dopamine has been shown to regulate cell proliferation in the SEZ (reviewed in Borta and Hoglinger (2007). Dopaminergic afferents form synapse-like structures with type C cells in the SEZ (Hoglinger et al., 2004). In addition, C cells express D2-like dopamine receptors, whereas migratory neuroblasts express receptors from both D1-like and D2-like receptors. The depletion of dopamine using neurotoxins such as MPTP (1-mthyl-4-phenyl-1,2,3,6-tetrahydropyridine) causes decreased proliferation of NPCs in both the SEZ and also the SGZ of the hippocampus (Hoglinger et al., 2004).

Decreased SEZ neurogenesis in Parkinson's disease has associated the loss of dopaminergic afferents to decreases in SEZ neurogensis and also to olfaction defects (Hoglinger et al., 2004). Indeed, Parkinson's patients report a diminished sense of smell before the onset of clinical motor symptoms (Berendse et al., 2001). This link has further been strengthened by the finding of a comparatively robust migratory pathway from the SEZ to the olfactory bulbs in the human forebrain (Curtis et al., 2007)

Serotonin

Serotonin (5HT) increases proliferation in both the SEZ and SGZ of mammals. Inhibition of 5-HT synthesis and selective lesions of 5-HT neurons of the raphe decrease proliferation (Brezun and Daszuta, 1999), whereas, fluoxetine-mediated inhibition of serotonin-reuptake increases proliferation in the dentate gyrus (Malberg et al., 2000).

Nitric oxide

The neurotransmitter nitric oxide (NO) is another neurotransmitter able to influence proliferation. In the SEZ of adult mice, neuronal precursors are found within the sphere of influence of a NO source and they express NO synthase (an enzyme that synthesises NO from L-arginine) at sites of terminal differentiation (Moreno-Lopez et al., 2000). Furthermore, administration of the NO donor DETA/NONOate to adult rats significantly increases both cell proliferation and migration in the SEZ and the DG (Zhang et al., 2001).

1.3.3.2 Growth factors and other extrinsic signals

Many different growth factors and other extrinsic signals influence the proliferation and differentiation of NPCs. A few examples are described below.

Growth factors, such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2), play a large role in the maintenance of adult NPCs. In vivo, both factors promote proliferation in the SEZ, and FGF2 increases the number of neurons in the OB (Kuhn et al., 1997). Although the infusion of FGF2 does not affect SGZ proliferation in mice, deletion of *Fgfr1* in the CNS decreases SGZ neurogenesis (Jin et al., 2003; Zhao et al., 2007). Therefore, FGF2 may be important for the maintenance of adult NPCs in the SGZ proliferation.

The neurotrophin brain-derived neurotrophic factor (BDNF) is another important positive regulator of adult neurogensis. Mice deficient in p75, a BDNF receptor, have a smaller olfactory bulb and decreased neurogenesis in the SEZ (Young et al., 2007).

For many of these extrinsic factors the target cells remain unknown. It is possible that they exert direct effects on NPCs themselves. However, it is also possible that the extracellular regulators could influence other cell types within the neurogenic niche, and thus exert an indirect effect on adult neural progenitors. For a complete list of extrinsic factors influencing adult neurogenesis see table 3.

Table 3. Extrinsic factors implicated in adult neurogenesis. Adapted from Simonovic (2008).

Factor	Function	References
BDNF	Enhances hippocampal proliferation and increases the number of BrdU-labelled cells. Can induce neurogenesis in non-neurogenic regions.	Katoh-Semba et al. (2002) ; Zigova et al (1998)
CNTF	Increases proliferation	Emsley et al (2003); Chojnacki et al. (2003)
EGF	Increases the number of EGF-responsive	Kuhn et al. (1997);

	precursors (transit amplifying cells).	Caille et al. (Caille et al., 2004); Doetsch et al. (2002)
EPO	Promote neuronal cell differentiation trough release of βNGF and BMP2 from astrocytes, associated with ERK-signalling pathway.	Park et al. (2006)
IGF-1	Supports survival and differentiation of NSC and promotes differentiation to oligodendrocytic lineage	DiCicco-Bloom et al. (1988); Drago et al. (1991); Åberg et al. (2000); Hsieh et al. (2004)
PDGF	Increases the proportion of neuronal cells in the absence of bFGF, acts on survival and proliferation of immature neurons. Maintains balance between neurogenesis and oligodendrogenesis.	Johe et al. (1996); Jackson et al. (2006)
PEDF	Secreted in the choroid plexus, increases neurogenesis	Ramirez-Castillejo et al. (2006)
TGF-β1	Arrests NSCs in G0/1-phase of the cell cycle	Craig et al. (2004); Enwere et al. (2004); Wachs et al. (2006)
VEGF	Secreted in the choroid plexus, increases survival of progenitors in culture. Increases proliferation of SEZ cells	Cao et al. (2004); Hashimoto et al. (2006); Schanzer et al. (2004); Jin et al. (2002)
Wnt3	Increase neurogenesis	Lie et al. (2005)
sAPP	Increases proliferation in adult SEZ precursors, supplied by the CSF.	Caillé et al. (2004)
Shh	Mitogen for neural stem cells	Machold et al. (2003)
RA	Induces neuronal differentiation in stem cells and supports survival of newborn neurons	Gajovic et al. (1997); Jacobs et al. (2006)
Extracellular nucleotides	Increase number of proliferating cells	Mishra et al. (2006)
Ephrin (Eph-A2)	Negatively regulate NSC proliferation	Holmberg et al. (2005)
Chemokines (activating CXCR4, CCR3)	Chemokines activating CXCR4, CCR3 inhibit neural precursor proliferation in isolated cells neurosperes and hippocampal slice culture by reduction of extracellular kinase phosporylation and increase of reelin expression. Chemokines promote survival of NPCs by maintaining them in quiescent state.	Krathwohl and Kaiser (2004)
GABA	GABA signalling between neuroblasts and astrocytes limit proliferation, thus contributing to the balance between amplification and mobilization of progenitors	Liu et al. (2005)
Dopamine	Decreases proliferation	Kippin et al. (2005)

Serotonin	Increases proliferation in hippocampus and SEZ	Brezun and Daszuta (1999); Malberg et al. (2000)
NO signalling	Decreases proliferation in SEZ	Packer et al. (2003); Moreno-Lopez et al. (2004)
Glucocorticoid hormones	Glucocorticoid receptors are expressed by NSCs and stimulation of the receptors in cell culture leads to reduced proliferation	Sundberg et al. (2006)
17β-estrogen	Increases the proliferation of embryonic but not adult NSCs.	Brännvall et al (2002), Martínez-Cerdeño et al. (2006)

1.3.3.3 Intracellular mechanisms

Several transcription factors play critical roles in postnatal neurogenesis. As shown by the phenotypes of their knockout mice, TLX, an orphan nuclear receptor, and Bmi-1 are necessary for the maintenance of adult forebrain NPCs (Molofsky et al., 2003; Shi et al., 2004). Pax6 promotes neuronal differentiation of SEZ progenitors, whereas Olig2 suppresses adult neurogenesis after brain injury (Buffo, 2007). Genes involved in epigenetic regulation, cell cycle regulation, DNA repair and chromosome stability are required for the proper function of adult NPCs. For a complete list of intracellular factors influencing adult neurogenesis see table 4.

Table 4. Extrinsic factors implicated in adult neurogenesis. Adapted from Simonovic (2008).

Protein	Function	Reference(s)
Bmi-1	Promotes self-renewal of NSCs, prevents senescence	Molofsky et al. (2003)
TLX	Maintains adult NSCs undifferentiated, proliferative	Shi et al. (2004)
Sox2	Attributes stem cell properties, required for proliferation and maintenance.	Ferri et al. (2004); Episkopou et al. (2005)
neudesin	Promotes neuronal differentiation in embryonic NSCs, inhibits differentiation of astrocytes and promotes self-renewal of NSCs expressed in neural precursor cells.	Kimura et al. (2006)
MELK	In vitro necessary for NSC proliferation, MELK expression is cell-cycle regulated, inhibition of MELK downregulates B-myb.	Nakano et al. (2005)

Msi-1	Maintains NSCs, activates notch signalling through translational repression of Numb (notch signalling repressor).	Sakakibara et al. (2001); Okano et al. (2002)
c-myb	Regulates NSCs proliferation and maintains ependymal-cell integrity, intrinsic markers of NSCs (sox2, Pax6) are reduced in NSCs lacking c-myb.	Malaterre et al. (2007)
β-catenin	β-catenin essential for maintenance and proliferation of neuronal progenitors and delays maturation of radial glial cells into intermediate progenitors.	Zechner et al. (2003); Wrobel et al. (2007)

The role of bHLH factors in adult neurogenesis

Whether the bHLH cascades used in the embryo are reused in adult neurogenesis remains a matter for investigation. There are however hints, in that some bHLH factors appear to have conserved roles in the adult brain. Mash1 is expressed in transient amplifying NPCs, as well as in neuroblasts, in the SGZ (Uda et al., 2007). The number of Mash1 positive cells can be increased through chronic treadmill running (Uda et al., 2007). Ngn2 is also expressed in immature neurons of the adult brain (Ozen et al., 2007).

Most proliferating zones in the zebrafish brain are almost completely overlapping with domains expressing proneural/neurogenic factors (such as delta genes and *ash1a*) or the Notch target *her4* (P. Chapouton, unpublished). These parallels suggest that adult neurogenesis events use molecular cascades reminiscent of those occurring in the embryo. Using a transgenic line expressing GFP under the control of the *her5* promoter Chapouton et al. (2006) have shown that *her5* is expressed in areas where adult NPCs can be found, such as at the isthmic proliferation zone (a population of slow dividing cells at the alar/basal junction between the midbrain and hindbrain). Recent studies have extended these results to show that *her3* and *her9* (C. Stigloher, unpublished), as well as a new factor, *her8a* (this study), are also expressed at the IPZ.

1.3.3.4 External factors influencing the rate of adult neurogenesis

The rate of adult neurogenesis is not static – it can be up- and down- regulated by many factors, including environmental enrichment (Kempermann et al., 1997), exercise (van Praag et al., 1999b), hippocampal dependent learning (Gould et al., 1999a), antidepressant drugs (Duman et al., 2001b), stress (Gould et al., 1997), glucocorticoids (Gould et al., 1991), ageing (at least partially due to glucocorticoid exposure) (Cameron and McKay, 1999) and drugs of abuse (see below).

Recently, the effect of psychostimulants on neurogenesis has become a focus in addiction research. This is firstly because drug-induced malfunction of the hippocampus may lead to the cognitive impairments affecting learning and memory seen in humans and animals exposed to drugs of abuse. Secondly, it's possible that alteration of hippocampal neurogenesis plays a role in the development of addiction-related behaviours (Eisch and Harburg, 2006). Although the focus of this work, the effects of drugs of abuse on adult neurogenesis are not restricted to psychostimulants. Chronic heroin, alcohol, morphine and nicotine have all be shown to reduce proliferation in the adult brain (Abrous et al., 2002; Eisch et al., 2000; Herrera et al., 2003).

Laboratory studies on the affects of psychostimulants on adult neurogenesis often produce differing and conflicting results, perhaps due to variations in experimental variables, such as species, dose and the length of administration and BrdU regime that was used. While several studies have reported that chronic cocaine (once daily for eight to 24 days) causes decreased proliferation, Eisch (2002) reports no change in proliferation after 17 days of twice daily cocaine (see table 5 for exact experimental conditions and references). There are no reports looking at the effects of chronic amphetamine on proliferation in the literature.

In order to affect adult neurogenesis, the administration of cocaine and amphetamine has to be chronic – studies looking at the acute affects of these drugs have failed to show an effect (Mao and Wang, 2001; Yamaguchi et al., 2004; Yamaguchi et al., 2005). In contrast, a single dose of methamphetamine has been shown to decrease proliferation in the gerbil (Teuchert-Noodt et al., 2000). It has been suggested that this is due to the longer half-life and more potent pharmacological effects of methamphetamine (Mao and Wang, 2001).

Table 5. An overview of the various studies looking at the effect of psychostimulants on the proliferation of NPCs in the adult brain.

Proliferation					
Drug	**Treatment**	**BrdU**	**Species**	**Conclusion**	**Reference**
Cocaine	2x daily over 17 days	day 18	Rat	No change in proliferation	Eisch, 2002
Cocaine	1x daily over 14 days	day 15	Rat	Decreased proliferation	Yamaguchi et al., 2004, 2005
Cocaine	1x 8 days	day 8	Rat	Decrease in proliferation	Dominguez-Escriba et al., 2006
Cocaine	1x 24 days	day 24	Rat	Decrease in proliferation	Dominguez-Escriba et al., 2006
Cocaine	1x	day 2	Rat	No change in proliferation	Yamaguchi et al., 2004, 2005
Amphetamine	1x	1 hour after drug	Rat	No change in proliferation	Mao et al., 2001
m-Amph	1x	concurrent with drug	Gerbil	Decreased proliferation 7 days post drug	Teuchert-Noodt et al., 2000
m-Amph	1x	36hrs after drug	Gerbil	No change in proliferation	Teuchert-Noodt et al., 2000
m-Amph	1x	70-76 days after drug	Gerbil	Decreased proliferation 7 days post BrdU	Hildebrandt et al., 1999

Table 6. An overview of the various studies looking at the effect of psychostimulants on the differentiation of NPCs in the adult brain.

Differentiation				
Drug	**Treatment**	**Species**	**Conclusion**	**Reference**
Cocaine	1x daily over 5 days	Rat	Increased numbers of immature neurons 2 and 4 days after cocaine. No change 6 hours or 1, 6 or 10 days after injections finished.	Mackowiak et al., 2005
Cocaine	1x	Rat	Decreased numbers of immature neurons at 1, 2, 4 and 6 days after cocaine. No change 6 hours or 10 days after single injection	Mackowiak et al., 2005
Cocaine	Constant over 7 days	Human fetal NPCs	Increased numbers of neurons	Hu et al., 2006

Reports on the effect of psychostimulants on maturation also vary (Hu et al., 2006). Mackowiak et al (2005) report that cocaine over five days can increase the number of immature neurons two and four days after drug administration has been ceased. In accordance, cocaine has been shown to increase differentiation of neural progenitor cells into neurons in cell culture (Hu et al., 2006). Mackowiak et al. (2005) find, however, no change at six hours or one, six or ten days after the last administration. In addition, they report that a single dose of cocaine decreased the number of immature neurons at several time points after cocaine (see table 6).

1.3.4 Significance of adult neurogenesis in brain plasticity

Addiction is a chronic disorder, and even after long periods of abstinence, the potential for relapse is high. This persistence is thought to be analoguous to learning and memory formation- both of these factors have links to adult neurogenesis, although the mechanism remains to be established (Berke and Hyman, 2000). Because of the links between drug abuse, adult neurogenesis and brain plasticity and also because of the results of our microarray experiments (see section '3.3 The molecular characterisation of the *nad* mutant' and Appendix 8.3), we decided to look at the effect of amphetamine administration on the proliferation and differentiation of NPCs in the zebrafish brain. The link between adult neurogenesis and amphetamine administration is further strengthened by the results of our microarray experiment, as many of the genes found to be changed upon amphetamine administration are expressed in known zones of proliferation in the zebrafish.

2. Aims & Achievements

The aim of my PhD thesis is to contribute to the understanding of neurogenesis control in the embryonic and adult vertebrate brain using zebrafish as a model organism. The maintenance of neuronal progenitors is a crucial process – premature differentiation only allows the formation of early-born cell types and causes disorganisation of the shape and cytoarchitecture of the brain. In addition, there is increasing evidence that neurogenesis plays a role in mood in the fully-developed, adult organism.

Previous work in our laboratory and in other groups has identified several members of the Hairy and Enhancer of Split-related (Her) family that actively inhibit neurogenesis in the neural plate. One of these is Her5 (Geling et al., 2003; Geling et al., 2004; Ninkovic et al., 2005), which is responsible for the maintenance of a progenitor pool at the midbrain hindbrain boundary. Although this previous work established Her5 as an important factor in the inhibition of neurogenesis, little was known about the factors upstream or downstream of the *her5* gene. Her proteins belong to the basic Helix-Loop-Helix (bHLH) family of transcription factors. These factors form dimers, either with themselves or with other bHLH factors, through which they act out their functions as either inhibitors or activators. The investigation about which binding partners interact with Her5 would further our understanding about its regulation and actions. To this aim I cloned and analysed the expression of many potential candidates from a yeast-2-hybrid (y2h) screen using Her5 as bait. One of these candidates was particularly promising, as it is strongly expressed at the midbrain-hindbrain boundary (mhb) at early embryonic stages. This candidate, *her8a*, is a novel member of the *her* family. I analysed the expression, function and regulation of *her8a*, showing that it is an inhibitor of neurogenesis, which varies in its ability to respond to Notch throughout development and in the adult. In addition, I have preliminary data that shows that *her8a* might be regulated by members of the *sox* family. This data is currently being prepared for publication. In addition, I also analysed the expression of *her8a* in the adult brain and compared its expression with that of other *her* family members. It is intended that this work will be incorporated into a joint manuscript with other members of our group comparing the expression patterns of *her* genes in the adult brain.

Recently, the maintenance of neuronal progenitors has been connected with the control of social behaviour. For instance, adult neural progenitors are required for the activity of some antidepressants, and many neurotransmitters involved in the control of social behaviour also affect neurogenesis. Our group has recently isolated a dominant zebrafish mutant, *no addiction* (*nad*^{dne3256}), which fails to respond to the psychostimulant amphetamine. I used this mutant as a tool to isolate the transcripts potentially mediating the rewarding affects of amphetamine in the brain. To this end, three separate microarray experiments were performed. The results of these experiments were analysed individually, and in addition they were also combined, in order to directly isolate the genes involved in reward – these genes make up our so called 'reward pool'. Gene ontology (GO) term enrichment analysis and network analysis were used to analyse the pathways causing the mutant phenotype, as well as those involved in the wildtype response to amphetamine. Although zebrafish is widely used and is becoming increasingly popular as a model organism, there are still far less publications on zebrafish than on, for example, mouse. As most network analysis tools rely on

information from the searches of abstracts and our 'reward pool' consists of a relatively small number of genes, commercially available software failed to form pathways of our results. Therefore, in collaboration with the 'computational modelling in biology' group headed by Fabian Theis, I helped develop a database that not only took information on zebrafish genes, but also the information from their mouse orthologues, into account. The bioinformatics analyses implement for the first time transcription factors involved in early brain development as transcriptional modulators of reward in the adult brain.

The results of the bioinformatic analysis were used to select the most interesting candidates, which were then validated using quantitative real time PCR and in situ hybridization. Five genes could be validated for the individual experiments. In addition, I performed qPCR experiments that distinguished whether the chosen candidate genes were responding to acute or chronic amphetamine administration. Lastly, the in situ hybridisation comparisons gave valuable information about the expression pattern of the chosen candidates. These genes were expressed in, among other regions, progenitor zones, further solidifying a link between adult neurogenesis and drug addiction.

The observation that many of the candidate genes that could be visibly down-regulated using in situ hybridisation were expressed in the ventricular zones (that is, areas of proliferation), led to a project in which I investigated the affect of amphetamine on proliferation and differentiation. I was able to show that chronic amphetamine administration can increase differentiation of progenitor cells into neurons. I also observed a trend towards decreased proliferation.

The questions that my work directly addressed are:

1. What are the upstream and downstream factors regulating the function of Her factors during neurogenesis control in the progenitor pools of the early neural plate?

2. What are the molecular mechanisms leading to addiction? Within this topic, I asked the following questions:

> What are the molecular mechanisms causing the lack of response of *nad* mutants to amphetamine?
>
> What are the molecular mechanisms specifically controlling reward in the adult brain?

3. What are the affects of amphetamine on the proliferation and differentiation of neural progenitor cells in the brain?

3. Results

3.1 Control of neurogenesis in the zebrafish embryo

Her5 has been shown to play a role in the maintenance of a progenitor pool at the zebrafish mhb. Her5 is a member of the bHLH superfamily of transcription factors. These proteins form homo- and hetero-dimers in order to carry out their functions as either repressors or activators of transcription, and information about which proteins form dimers with Her5 would give us valuable information about its actions. As a starting point for this project, I had a y2h performed in order to identify binding partners of Her5, which identified a novel bHLH factor, Her8a. A manuscript of this work is currently being prepared for publication.

3.1.1 Possible binding partners of Her5 as revealed using yeast-2-hybrid

In order to recover binding partners of the Her5 protein, a yeast 2 hybrid screen was performed in which a 181 amino acid fragment of Her5 (the basic domain and the WRPW motif were removed, see also figure 28, A) was screened against an 18-20hpf embryo zebrafish library. This screen returned 280 positive clones, from a total of 76.1 million tested interactions. These 280 positive clones represented 75 unique protein-protein interactions. The quality of these interactions was graded using a PBS scoring system – a confidence source based on two factors: firstly, a local score takes into account the redundancy and independency of prey fragments, as well as the distribution of reading frames and stop codons in overlapping fragments. Secondly, a global score takes into account the interactions found in all the screens performed at the company where the screen was performed, Hybrigenics, using the same library. The assigned confidence scores were divided into five categories relating to the level of confidence: A = highest; B = very good; C = good; D = low and N/A = no score could by assigned. Our screen returned 6 As, 9 Bs, 2 Cs, 49 Ds and 9 N/As (see 'Appendix 8.1: Summary of yeast-2-hybrid results' for a detailed description of all recovered candidates). As further indication of the validity of the assay, we note that Her5 was found to bind with Her11 with a score of B, an interaction that had been shown previously in our laboratory (Ninkovic et al., 2005).

From the proteins assigned from A to C, I chose seven to investigate further, based on score and – if available – gene expression pattern (gene expression should overlap with that of *her5* at early segmentation stages (in this study I concentrated on the three somite stage), but not be ubiquitous), functional data in zebrafish and information on orthologues (with emphasis on cell cycle control). The chosen genes were *Catenin, beta like 1* (*ctnnbl1*), *hairy-related 8a* (*her8a*), *hairy-related 13* (*her13*) (formerly *her13.1*), *Hairy and enhancer of split 6* (*hes6*) (formerly *her13.2*), *protein phosphatase 1G* (formerly *2C*) (*ppm1g*), *prosaposin* (*psap*) and *tpr* (tetratricopeptide repeat)-*like* (*tpr-like*).

As a first step, I cloned these genes into pCRIITOPO vector and performed in situ hybridisation on wildtype embryos at various stages. *ctnnbl1* and *tpr-like* were expressed ubiquitiously throughout the embryo at 3 somites, 24hpf and 48hfp (Fig. 25, A-B shown for 24hpf; other stages unshown). *ppm1g* is expressed ubiquitously at three somites (Fig. 25, C).

At 24hpf it is also broadly expressed throughout the embryo, expression appearing denser in the telencephalon, midbrain-hindbrain and in the eye. By 48hpf the expression is restricted to the proliferation zones, in particular in the retina and in the tectum (Fig. 25, D-E). *psap* is expressed in blood cells and in the pronephric ducts (Thisse et al. (2001) and confirmed in our findings) and also in the brain (Fig. 25, F).

Three of the chosen proteins belong to the same family as Her5 – Hes6, Her8a, and Her13. In situ hybridisation revealed that *her13* is expressed in a sub-set of proneural clusters within the presumptive spinal cord at three somites (Fig. 25, G). Expression is lost by 11 somites, before being regained by 24hpf (Fig. 25, H). *her13* expression did not overlap with that of *her5* at our stage of interest – three somites – and its expression indicates that it is not involved in the maintenance of progenitor zones. *hes6* has been implemented in somite formation (Kawamura et al., 2005). At 24 hpf *hes6* is expressed in the retina and in the central nervous system (Thisse et al. (2004) and confirmed in Fig. 25, J). At earlier stages, *hes6* is expressed in the tail bud and posterior paraxial mesoderm – its expression does not overlap with that of *her5* at the three somtie stage (Fig. 25, I). The last gene cloned from this subset of *Drosophila* hairy/E(spl) family genes, *her8a*, has a broad expression pattern in the early embryo, which becomes restricted to the ventricles by 48hpf. In the early embryo *her8a* has a broad expression pattern, which becomes restricted to the ventricles by 48hpf. Before tailbud the expression is weak to undetectable (Thisse and Thisse, 2004). At tailbud *her8a* is expressed throughout the embryo, with the exception of the eye field and the midline (Fig. 26, A). It is more densely expressed in a broad area surrounding the midbrain hindbrain boundary (mhb) (indicated with black arrow). At 10 somites *her8a* is expressed in stripes in the hindbrain (Fig. 26, B-C). There is denser expression in rhombomere 1 (Fig. 26, C). At 24hpf *her8a* is expressed throughout the central nervous system; expression is weaker at the mhb and at the zona limitans (Fig. 26, D). Denser expression can be observed anteriorly in rhombomere 1 (Fig 26, E). Cross sections of the brain at 24hpf reveal that *her8a* is expressed centrally in the embryo, whereas Hu is expressed outwardly in differentiating neurons (Fig. 26, G). The expression of *her8a* overlaps with that of the neuronal marker Hu in a few single cells at the boarder of these domains (Fig. 26, H). From 48hpf through to adult *her8a* is expressed in ventricular zones throughout the brain, as shown here at the mhb in the adult (Fig. 26, I-M) (details are shown in section: '3.2 *her* gene expression in the zebrafish adult brain', and will be published separately else where). At early segmental stages, the expression of *her8a* overlaps with that of genes expressed in progenitor zones, such *her3* and *her5*, as well as that of proneural genes (Fig. 27, A-C).

Of the six genes chosen for further investigation, one gene – *her8a* – fulfilled the requirement of a non-ubiquitous expression pattern that overlaps with that of *her5* at 3 somites. Although not within the scope of this study, which focuses on early patterning, the other genes may prove to be interesting candidates that work with *her5* at other stages. In particular, *hes6* and the previously uncharacterised *ppm1g* are especially promising, as they are expressed in the ventricular zones of embryos at 48hpf.

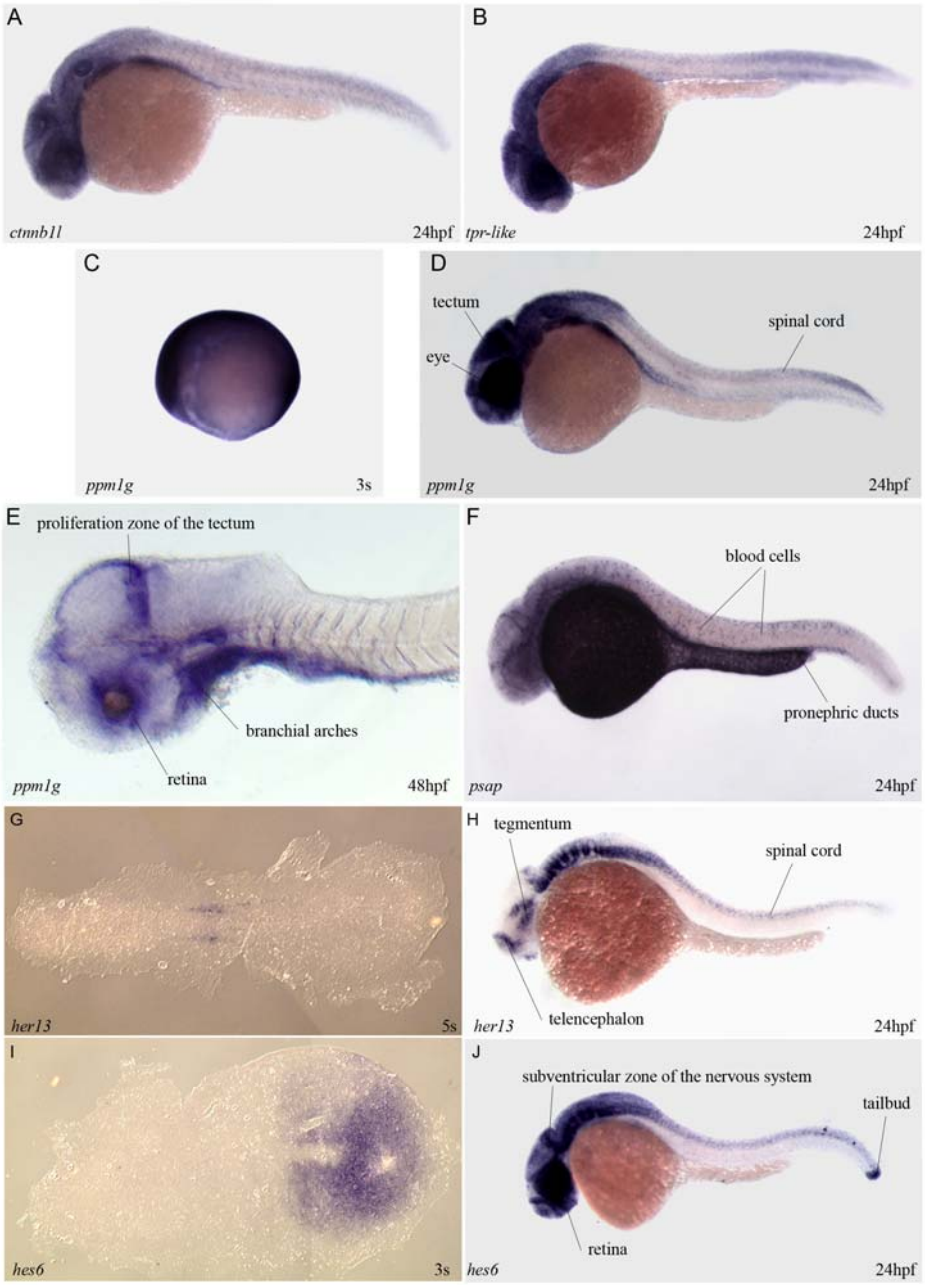

Figure 25. In situ hybridisation revealing the expression of candidates recovered in a yeast-2-hybrid screen for a Her5 fragment. *ctnnb1l* and *tpr-like* are expressed ubiquitously throughout early embryonic development (shown here at 24hpf). *ppm1g* is expressed ubiquitously at three somites (C) and at 24 hpf (D). Expression is stronger in the tectum and in the eye. By 48hpf *ppm1g* is expressed to the proliferation zone of the tectum and the retina and in the branchial arches (E). *psap* is expressed in blood cells and in the pronephric ducts (F). At five somites *her13* is expressed in some proneural clusters of the presumptive spinal cord (G). Its expression is lost at 11 somites, before being regained at 24 hpf (H). *hes6* is expressed in the tail bud at three somites (I) and throughout the nervous system and the retina at 24 hpf (J).

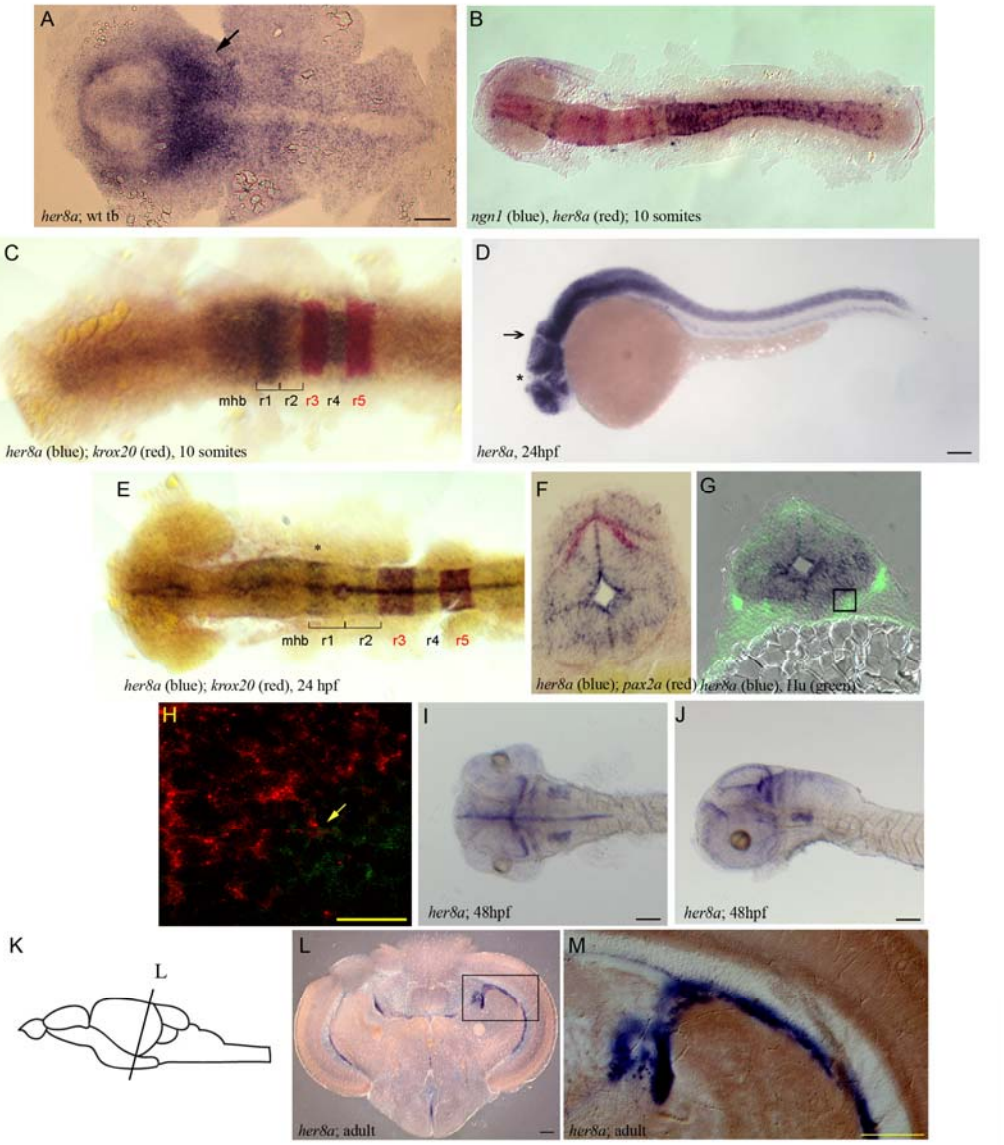

Figure 26. *her8a* is at first expressed broadly throughout the embryo in both progenitor and proneural zones, before later becoming restricted to progenitor areas. (A) *her8a* at tailbud stage. It is expressed throughout the embryo with the exception of the eye field and midline. The expression is stronger at the midbrain-hindbrain boundary (mhb) (black arrow). At 10 somites there is a denser stripe of *her8a* expression at rhombomere 1 (B-C; see *). At 24hpf *her8a* is expressed broadly throughout the embryo (D-H). Expression is weaker at the mhb ((black arrow) and zona limitans (*) in (D) and *her5* expression (F)). Cross sections through the brain at 24hpf with *her8a* in blue and Hu in green reveal that they are found in almost mutually exclusive areas (G). Confocal images of equivalent sections of G, with *her8a* stained with fast red and Hu immuno in green, reveal double labeling of a few individual cells at the border (H). Yellow arrow points to a double labeled cell. By 48hpf *her8a* is expressed solely in the ventricular zones (I-J). This is continued in the adult, as shown here in the midbrain (L-M; K indicates level of cross section). Black lines = 100µm; yellow bars = 25µm.

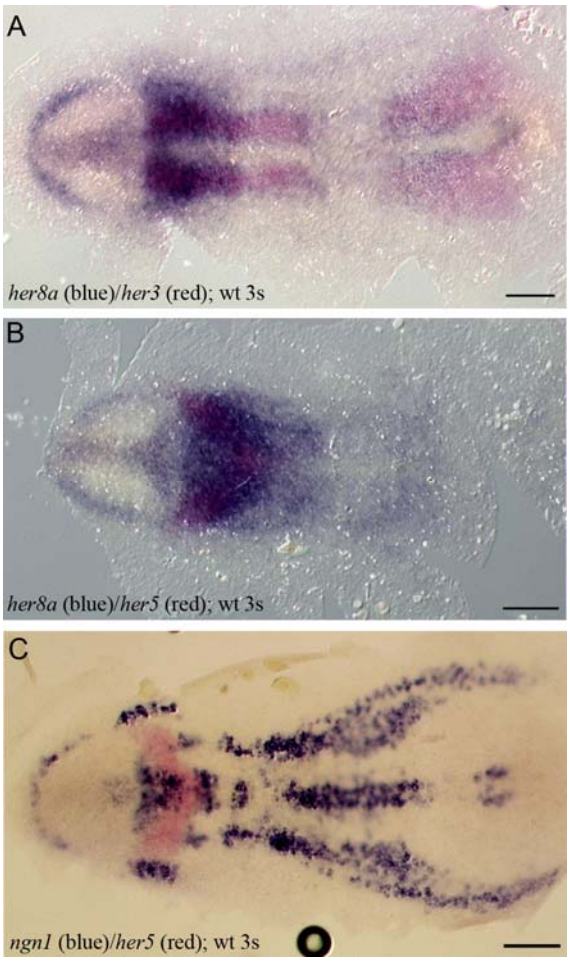

Figure 27. *her8a* is expressed in both neurogenic and progenitor zones. The expression of *her8a* overlaps with that of *her3* (A) and *her5* (B) – genes that are expressed in progenitor zones. Using a photo of a double staining of *neurog1* and *her5* shows for comparison, it can be seen that *her8a* expression also overlaps with that of *neurog1* (C).

3.1.2 Her8a is a negative regulator of neurogenesis that responds to Notch in a context-dependent manner

From the expression analysis described above I decided to concentrate on the functional analysis of Her8a.

3.1.2.1 Her8a belongs to the Hairy-E(spl) family

her8a is 2284 bp long, encoding 221 amino acids. It encodes a bHLH and an orange domain and has a WRPW motif at its C-terminus - characteristics common to the *Drosophila* hairy/E(spl) family. Its sequence is most similar to the zebrafish proteins Her13 and Hes6. Its nearest mouse homologue is Hes6. Her13, Hes6 and Her8a, as well as the mouse Hes6, exhibit a shortened loop when compared to other hairy-E(spl) members, such as Her5 and Her11 (see figure 28). The loops of Her13 and Hes6 contain 5 less amino acids when compared with other members; the loop of Her8a has 3 less amino acids (Gajewski et al., 2006 and Figure 28).

A

B

```
Dr_Her8a        ------MTASNMGNGPEKNFNAKEERKLRKPLIEKKRRERINSSLEQLKG
Dr_Her5         -------------------MEQKDMRRVPKPLMEKRRRDRINQSLETLRM
Dr_Her3         ---MAAASNSAATAKPQN------VKKVSKPLMEKKRRARINKCLNQLKS
Dr_Her11        ----------MKSTPTFNMTKTEGIKRRLKPVIEKKRRDRINHNLDALRD
Dr_Her13        -MVHAAELRNNLGVGEYACYGFKGDRKTRKPIVEKKRRARINESLQDLRT
Dr_Hes6         ---MAPASRSNT--HDEDNYGIK-DRKTRKPLVEKKRRARINESLQELRL
Mm_Hes6         MAPSQAPSRDRAGQEDEDRWEARGDRKARKPLVEKKRRARINESLQELRL
                                         ::  **::**:** ***  *: *:

Dr_Her8a        IMVDAYN---LDQSKLEKADVLEITVQHMENLQRGHGQGGSNSPGTGFES
Dr_Her5         LLLENTNNEKLKNPKVEKAEILESVVHFLRAEQASETDPFQITRVKRART
Dr_Her3         LLESACSN-NIRKRKLEKADILELTVKHLRHLQNTKRG------LSKACD
Dr_Her11        LLFKNTADTRLQNPKLEKAEILDLAVQYIKKTIRKTETARNSNQMDCKST
Dr_Her13        LLTNND-----LQTKMENAEVLELTVKRVESILQSRSQ---ETGTVTQEA
Dr_Hes6         LLADPD-----AQVKMENAEVLEMTVKRVESILQNKAK---EADSVNREA
Mm_Hes6         LLAGTE-----VQAKLENAEVLELTVRRVQGALRGRAR---EREQLQAEA
                ::          : *:*:*::*: .*: :.

Dr_Her8a        RQR----------YSSG----------YIQCMHEVHNLLLSCPGMD
Dr_Her5         EESDEDVESPCKRQSYHDG----------MRTCLLRVSNFITGKSHEFG
Dr_Her3         SAE----------YHAG----------YRSCLNTVSHYLRAS-DTD
Dr_Her11        QNQ----------FVISPAGPLYTSDYCRRFKTSEQNEVLLNLGVSQN
Dr_Her13        SER----------FAAG----------YIQCMHEVHTFVSTCPGIE
Dr_Hes6         NER----------FAAG----------YIQCMHEVHTFVSSCPGID
Mm_Hes6         SER----------FAAG----------YIQCMHEVHTFVSTCQAID
                                                    .

Dr_Her8a        KTLGARLLNHLLKSLPHISTEPSGTSSAGTSSPLPLSP----------
Dr_Her5         QELEKACENI----------HKSHSRQVQLLSTPS----------
Dr_Her3         RDSRSIMLTNLTSGLNHNRVPDFSTVESDPALIFTLPST------LRRPH
Dr_Her11        LSGSSKTGNKLVSQKGFLLSPPGQNYHQELLLHPESSLYGSSLLRQSTSP
Dr_Her13        ARVAAELLNHLLESMPLN-ENHLHEMIKDLISEPSSSGD------SWQSG
Dr_Hes6         ATIAADLLNHLLECMPLNDEERFQDILSDLISDSNNSG-------TWP-G
Mm_Hes6         ATVSAELLNHLLESMPLREGSSFQDLLGDSLAGLPGGS----------
                        .:

Dr_Her8a        -----------TQSPINLPSSLQPHALHLSPSPPSSPTHSLVRPREQFSP
Dr_Her5         ------------LIEPQVHLYEDPSQQHLAHVQLSNSCTPSGCSKLAQRT
Dr_Her3         KVPIRTDVSYSSFQQTAERKVCLMPKRTEIGDSDRMSLDAALRSQESK-K
Dr_Her11        SISSSSQYSSPPSSPTFTSTSCSPSSSPPCPSTSCAAFPDQLSPLITPLS
Dr_Her13        EAQ----NPGRHCVS-GMSSELPQFLSNPFCDDLCSDLDET-ETEER--S
Dr_Hes6         EAAYATLSPGGTSVANGGSSALSPAPSTTSSDDICSDLDDT-DTEHSRIS
Mm_Hes6         ---------GRSSWPPGGSPESPLSSPPGPGDDLCSDLEEIPEAELNRVP
                                                          .

Dr_Her8a        PSSPSPQSPASL------------------PPFFPGVDPSMWRPW-
Dr_Her5         VPAMTSSPKQP----------------------VMLCDPVWRPW-
Dr_Her3         AETTHFRPKDLK------------------VIECCIFKQNYWRPW-
Dr_Her11        LQRPVFVPQAILPHMTRDLTPPHSPVLALRQDPFPLPNQHAWRPWS
Dr_Her13        ASAEDTLDFSM----------------------VTHS-KFMWRPW-
Dr_Hes6         VDAGDQAPVVP----------------------TLYTNKSIWRPW-
Mm_Hes6         AEGPDLVSTSLG------------------SLTAARRAQSVWRPW-
                                                    ****
```

Figure 28 (see above). Yeast-2-hybrid screen using Her5 as bait recovered Her8a as a candidate binding partner. A) A 181 amino acid fragment of the basic-helix-loop-helix protein Her5 (see red box) was fused with a lexA binding domain. B) A novel member and potential binding partner of Her5 – Her8a – was recovered in the yeast-2-hybrid screen. This protein exhibits the typical characteristics – bHLH, orange domain and a WRPW motif - of hairy/E(spl) family members. Her8a belongs to the Hes6 subgroup of hairy/E(spl) transcription factors This subgroup is characterized by a shortened loop. The loop of Her8a has three less amino acids than other members of the family. The loops of zebrafish Her13 (previously Her13.1), zebrafish Hes6 (previously Her13.2) and mouse Hes6 have five less amino acids than other family members (see alignment and Gajewski et al. (2006).

3.1.2.2 The expression of *her8a* transitions from being Notch-independent to Notch-dependent during early development

Although many E(spl) transcription factors are downstream effectors of Notch signalling, previous work has shown that zebrafish members of the *hairy/E(spl)* family that are expressed in progenitor zones, such as *her5* (Geling et al., 2004)**,** *her11* (Jovica Ninkovic, personal communication), *her3* (Bae et al., 2005) and *her9* (Latimer et al., 2005), do not require Notch for their expression. This is in contrast to other members of the zebrafish *hairy-E(spl)* family (such as *her4*) that are expressed in neurogenic zones, in a salt and pepper pattern, alternating with cells expressing proneural genes such as *neurog1***.** Unusually, *her8a* is expressed in both proliferation and neurogenic zones; its expression covers that of proneural genes such as *her4*, but also that of genes expressed in progenitor zones, such as *her5*. We thus decided to see whether *her8a* is dependent on Notch for its expression. DAPT, an inhibitor of γ-secretase, blocks Notch by preventing the cleavage of NICD and has a strong neurogenic effect (Crawford and Roelink, 2007; Geling et al., 2002). As anticipated from previous studies (Geling et al., 2002), DAPT treatment increased the amount of *neurog1* positive neurons in the proneural clusters (Fig. 29 A-B). At 3 somites, DAPT treatment did not affect the expression of *her8a*, indicating that Notch is not required for its expression at this early stage (Fig. 29, C-D).

However, by 48hpf *her8a* expression was completely down-regulated by DAPT treatment (Fig. 29, E-F). This indicates that *her8a* transitions from being Notch insensitive to Notch sensitive throughout development. Interestingly, we also found that the requirement of Notch varies according to domain for *her4*. We found that at 48hpf DAPT can inhibit the expression of *her4* in the spinal cord, while causing an upregulation in the eye field. The expression in the telencephalon is not affected (Fig 29, G-H). Thus it appears that, at 48hpf, while Notch is still required for the expression of *her4* in the spinal cord, it is at the same time directly or indirectly inhibiting the expression of *her4* in the eye field.

In addition to not requiring Notch for their expression, genes of the progenitor zones, such as *her5* and *her3*, differ from other family members in that they are inhibited rather than activated by Notch overexpression, whereas the expression of *her9* remains unchanged (Bae et al., 2005; Geling et al., 2004). In contrast, NICD overexpression activates the expression of *her4* throughout the embryo (Yeo et al., 2007). In order to investigate to which category *her8a* belongs, we injected capped RNA for the Notch intracellular domain (NICD)

(62.5ng/µl) and tested the expression of *her8a* via in situ hybridisation. Overexpression of NICD causes ectopic expression of *her8a* throughout the embryo (Fig. 29, I-J), suggesting, that while Notch is not needed for *her8a* expression, it can be activated by Notch under non-physiological conditions. The ectopic expression caused by overexpression of NICD produced less dense ectopic expression of *her8a* at the caudal end of the neural plate (indicated in Fig. 29 with an *). In order to elucidate whether this was caused by differences specific to *her8a* or a general decreased ability of this area to respond to NICD overexpression, we looked at the expression of *her4* in NICD overexpressed embryos. We found that NICD overexpression also causes less dense ectopic expression of *her4* caudally, as does *her8a,* indicating that this difference is not specific to *her8a* (Fig. 29, K-L). As a control, we also tested that we were capable of down-regulating *neurog1* expression through NICD overexpression (Fig. 29, M-N).

Together, it appears that the responsiveness to Notch is not fixed, but changes according to developmental stage and the domain of supposed expression and thus according to cellular context. The ability of Notch to artificially activate expression of *her8a* at a time point at which *her8a* does not rely on Notch for its expression is retained.

3.1.2.3 Overexpression of *her8a* perturbs *neurog1* expression

Embryos were injected with *her8a* capped RNA encoding the full length protein at the one cell stage. They were subsequently fixed at 3 somites for in situ hybridisation. *her8a* overexpression causes a complete loss of *neurog1* expression throughout the embryo (Fig. 30, A-B). This shows that *her8a* is capable of inhibiting *neurog1* at non-physiological concentrations. In the wildtype embryo, *neurog1* and *her8a* are expressed in the same domains. It is possible that the amount of *her8a* needed to prevent *neurog1* expression exceeds that of physiological conditions. As the morpholino mediated knockdown of *her8a* produces the same phenotype as *her3* knockdown, we investigated whether the overexpression of *her8a* activates ectopic *her3* expression. The expression of *her3* was not affected in embryos with overexpressed *her8a* (n=29; staining not shown). Thus, under artificial conditions of overexpression of *her8a* can cause a loss of *neurog1*, even though the expression of *her8a* overlaps with that of *neurog1*.

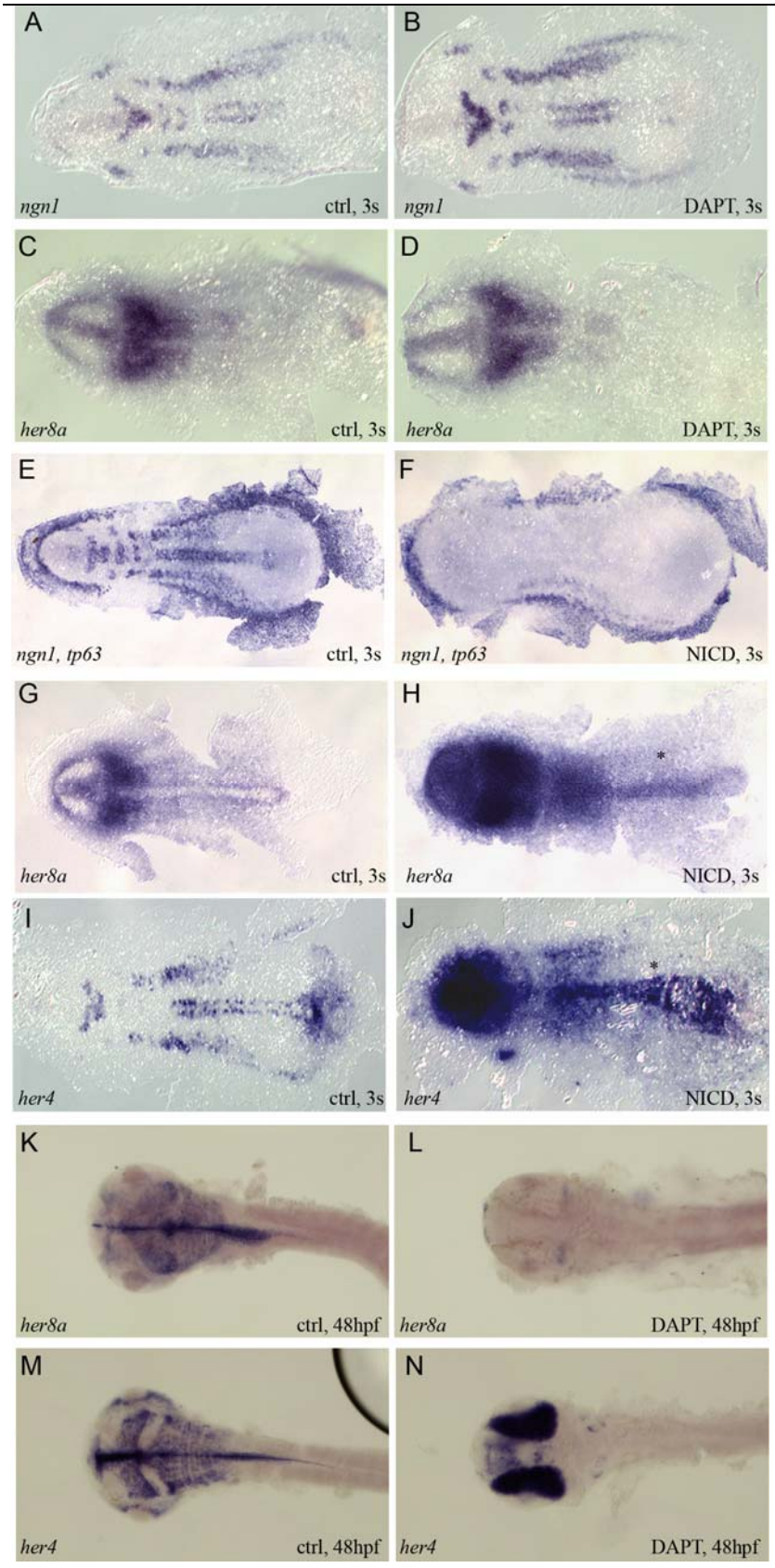
A
ngn1
ctrl, 3s
B
ngn1
DAPT, 3s
C
her8a
ctrl, 3s
D
her8a
DAPT, 3s
E
ngn1, tp63
ctrl, 3s
F
ngn1, tp63
NICD, 3s
G
her8a
ctrl, 3s
H
her8a
NICD, 3s
I
her4
ctrl, 3s
J
her4
NICD, 3s
K
her8a
ctrl, 48hpf
L
her8a
DAPT, 48hpf
M
her4
ctrl, 48hpf
N
her4
DAPT, 48hpf

Figure 29 (see above). *her8a* transitions from being Notch-independent to Notch-dependent during early development. In a first experiment, embryos were treated from 50% epiboly to three somites with 100µm DAPT. As shown in previous work, DAPT treatment greatly increases the number of *neurog1*-positive cells within each proneural cluster (A-B). *her8a* expression was unaffected in DAPT treated embryos at three somites (C-D). Overexpression of the notch intracellular domain (NICD) results in the overexpression of *her8a* throughout the embryo (G-H). This is similar to the effect on *her4* (I-J). NICD overexpression decreased *neurog1* expression throughout the embryo, as previously published (E-F) (Geling et al., 2004). DAPT treatment from 24hpf to 48hpf completely eliminated *her8a* expression throughout the embryo (K-L). This is in contrast to *her4*. The same DAPT treatment decreased the expression of *her4* in the spine and increased the expression in the eye. The expression in the telencephalon remained unaffected (M-N).

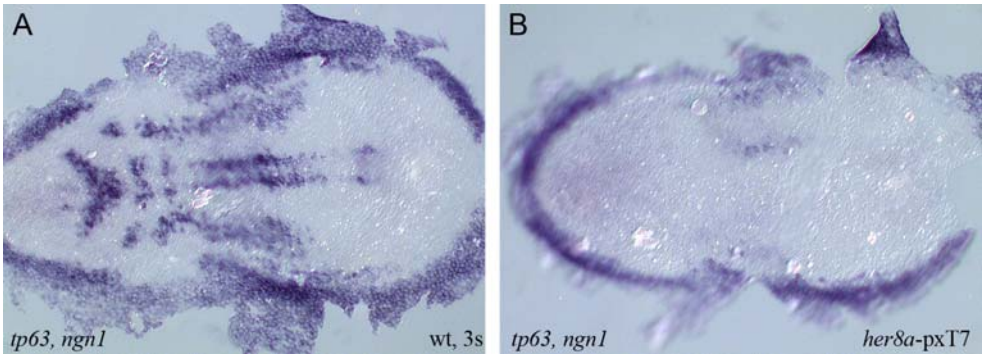

Figure 30. Overexpression of *her8a* causes a loss of *neurog1* expression throughout the embryo. Full length *her8a* was cloned into pXT7 and capped RNA from the construct was injected into embryos at the one cell stage. Subsequent staining with *neurog1* revealed that *her8a* causes a loss of proneural zones. *tp63* was used to delimit the neural plate.

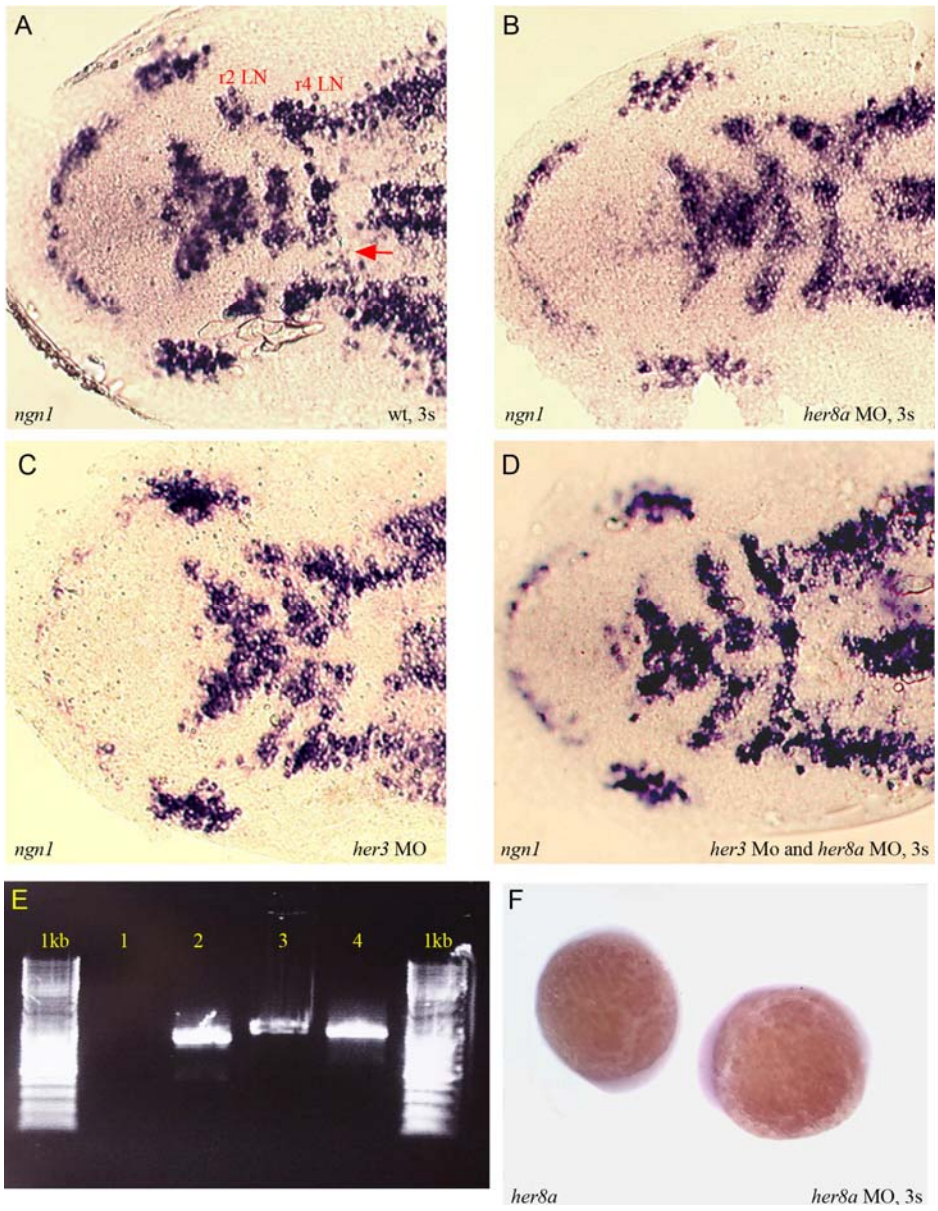

Figure 31. Morpholino-mediated knockdown of *her8a* leads to the ectopic expression of *neurog1* at rhombomeres 2 and 4. Injection of a splice morpholino against *her8a* at the one cell stage leads to ectopic expression of *neurog1* at 3 somites as revealed here using in situ hybridization and compared to the non-injected control (red arrow on control indicates area normally free of *neurog1* expression) (A-B). This resembles the previously published phenotype of *her3* knockdown (C). Combined knockdown of *her3* and *her8a* does not produce any additional phenotype (D). Injection of the *her8a* splice morpholino at the one cell stage leads to an almost complete reduction of *her8a* expression at 3 somites, as revealed by RT-PCR and in situ hybridization (E-F); 1=*her8a* on morpholino injected embryos, 2=*her8a* on uninjected embryos, 3=*sox1b* on morpholino injected embryos and 4=*sox1b* on uninjected embryos. The expression of other early markers, such as *sox19b*, is not affected.

In order to further elucidate the action of *her8a*, we removed the basic domain from *her8a* (*her8a*deltab). This removed the ability of *her8a* to repress *neurog1* transcription (not shown). The function of the basic domain is to bind DNA, indicating that this is necessary for the *her8a*-mediated repression of *neurog1*. Other members of the family, such as *her3*, also require their basic domain for the repression of *neurog1*. Our experimental evidence indicates that *her8a* is acting as a repressor; however, it is unclear whether *her8a* acts directly or indirectly as an activator of other genes that in turn would repress *neurog1*. In order to investigate this, we made constructs with either two copies of the transactivation domain of VP11 (*her8a*VP11x2) or the repressor domain of Engrailed (*her8a*eng) fusioned at the C-terminal end. Neither of these constructs could suppress nor activate *neurog1* expression. This indicates that the addition of these domains changes the structure of *her8a* so that it can no longer act on *neurog1*.

3.1.2.4 *her8a* knockdown phenotype resembles that of *her3*

Embryos were injected with a splice morpholino against *her8a* and fixed at 3 somites. In situ hybridisation shows that this causes the ectopic expression of *neurog1* between rhombomeres 2 and 4 (Fig. 31, A-B). The *her8a* knockdown phenotype resembles that of *her3* knockdown (Fig. 31, C), suggesting a functional interaction. One possibility is that the two genes can compensate for each other and that the knockdown of both genes is required to produce a phenotype of a greater magnitude than the previously described ectopic expression of proneural markers in rhombomeres 2 and 4. The simultaneous injection of morpholinos against *her8a* and *her3* (at the same combinations shown to produce the individual phenotypes) produces no additional phenotype (Fig. 31, D), indicating that the genes are not compensating for each other in other areas of the embryo.

Double in situ reveals that the area of ectopic neurogenesis caused by *her8a* or *her3* knockdown is where the intense expression of *her8a* overlaps with that of *her3*, but not at the midbrain-hindbrain boundary (mhb). In order to test whether *her8a* or *her3* could be working up- or downstream of each other we tested whether *her8a* knockdown could affect *her3* expression and vice versa. We found that the morpholino mediated knockdown of *her8a* did not change the expression pattern of *her3* (n=34) and vice versa (n=24). Furthermore, combined knockdown of *her3*, *her5*, *her9* and *her11* did not change *her8a* expression (n=28). Therefore, it appears that *her8a* and *her3* are not influencing each other at the level of transcription.

As the area affected by *her8a* knockdown is relatively small compared to its broad expression at three somites. This parallels the knockdown phenotypes of other members of the family, such as *her3* (Hans et al., 2004), where the induction of ectopic neurons is small compared to the expression of the gene. In order to further substantiate this observation, reverse transcription PCR was used to reveal an almost complete lost of expression of *her8a* due to the morpholino knockdown at three somites, whereas other genes remained unaffected (Fig 31, E). The lost of *her8a* expression was confirmed using in situ hybridisation (Fig. 31, F), indicating that *her8a* is required for its own expression either directly or indirectly as part of a feedback loop.

3.1.3 Sox family members as possible upstream factors of Her8a

A scan of the *her8a* promoter (100bp downstream and 1000bp up-stream of the atg) with ModelInspector (Genomatix) (Frech et al., 1997) revealed a potential Sox (Sry-related HMG box) binding site (SORY_OCT1_01) at 873-897(+). ModelInspector uses Genomatix's in-house Promoter Module Library, which includes experimentally verified models for functional subunits of promoters. In this case the promoter sequence was derived from a publication in mouse describing the activation of the FGF4 enhancer by Sox2 and Oct-3 (Ambrosetti et al., 1997). This led us to investigate the possibility that a member of the Sox gene family is (partially) controlling *her8a* expression.

Sox genes encode a family of transcription factors, many of which play a role in development. Mouse *Sox2* is a member of the Group B, a division of *Sox* genes that appear to be involved in neural development, starting from the early derivation of the neural primordium to the eventual differentiation of the central nervous system (Okuda et al., 2006). Group B genes can be divided into two subgroups – Group B1 and Group B2. Groups B1 and B2 share highly conserved HMG domains, but their sequences differ outside this domain. Sox2 is part of Group B1. I chose to investigate zebrafish genes from Group B1 (*sox2* (Okuda et al., 2006), *sox3* (Okuda et al., 2006), *sox19a* (Thisse et al., 2001) and *sox19b* (Thisse et al., 2001) (see Okuda et al. (2006) for phylogenetic description). In addition, I also investigated *sox21b* (previously *sox21*), a member of Group B2, as *sox21b* has expression specific to the midbrain-hindbrain boundary (mhb) at early embryonic stages (Rimini et al., 1999). Although *sox1a* and *sox1b* are also members of Group B1, I did not investigate them further, as they are not expressed at the mhb at early embryonic stages (Okuda et al., 2006; Thisse and Thisse, 2004).

The first task was to compare the expression of the chosen candidate genes with that of *her8a*. I cloned *sox2*, *sox3*, *sox19a*, *sox19b* and *sox21b*. Where possible I compared their expression using double in situ hybridisation (Fig. 32, A-D). For *sox21b* no nice double staining was obtained, so its expression is compared aside that of *her8a* (Fig. 32, E-F). *sox2* and *sox3* are expressed in a stripe posteriorly in the zone of dense *her8a* expression surrounding the mhb (see blue arrows). *sox19a* and *sox19b* expression covers the area of dense expression of *her8a* at the mhb entirely. In addition, *sox2*, *sox3*, *sox19a* and *sox19b* have similar expressions to *her8a* in the telencephalon and ventral diencephalon and they are also all excluded from the eye field. As previously published, *sox21b* is expressed at the mhb at 3 somites (Rimini et al., 1999) (Fig 32, E).

In summary, the expression of all of the selected Sox family member candidates overlaps with that of *her8a*. In a next step morpholinos were designed in order to knockdown the expression of the selected *Sox* genes. Morpholinos were used to knockdown *sox19a*, *sox19b* and *sox21b* individually. A single morpholino was designed for *sox2* and *sox3*, as they share the sequence surrounding their ATGs, in an attempt to reduce toxicity should a combined knockdown be necessary. These morpholinos were injected in progressively higher concentrations (starting at 0.5 mM) until toxicity was reached (usually at around 2mM). The embryos were fixed at 3 somites and stained for either *her8a* or *neurog1* expression using in situ hybridisation. None of the morpholinos produced a phenotype for either of the two genes expression. It is possible that gene members of the same family fully or partially compensate for each other, if the expression of one of them is knockeddown. As a consequence, I

knockeddown all five genes at once by injecting a mixture of the morpholinos (*sox2/3* (1mM), *sox19a* (1mM), *sox19b* (0.25mM) and *sox21b* (1mM)). The resulting embryos had reduced *her8a* staining at three somites (Fig. 33, A-D). They also had ectopic *neurog1* staining in the mesoderm (Fig. 33, E-F). I attempted to let these embryos develop longer, to see whether there is also ectopic expression of *neurog1* at the midbrain-hindbrain area. However, the resulting embryos did not have an *neurog1* phenotype, so it is possible that the combined knockdown embryos do not develop beyond this point or the morpholino losses its activity.

In a reverse step, I also looked at the effect of *her8a* knockdown on the expression of *sox2*, *sox3*, *sox19a*, *sox19b* and *sox21a*. No effect could be observed (see table 7), indicating that Her8a acts downstream of *sox* and there is no feed-back loop.

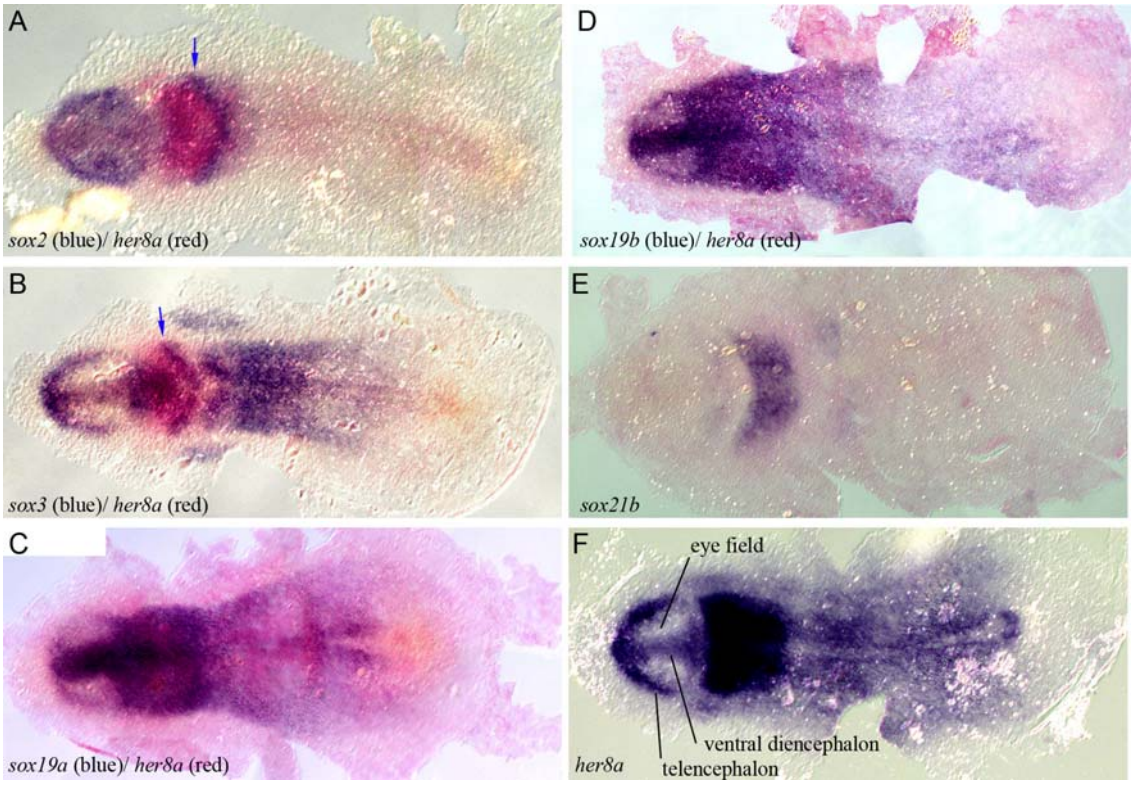

Figure 32. The expression of *sox* family members overlaps with that of *her8a*. All expression revealed using in situ hybridisation (blue and red stainings). The expression of *her8a* overlaps with that of *sox2* (A), *sox3* (B), *sox19a* (C) and *sox19b* (D) (all double in situ hybridisations with *her8a* in blue). *sox2* and *sox3* are expressed in a stripe posterior in the dense expression of *her8a* surrounding the midbrain-hindbrain boundary (mhb) (see arrows). *sox21b* is shown singly, with *her8a* underneath for comparison (E-F). The expression of *sox21b* overlaps with that of *her8a* at the mhb.

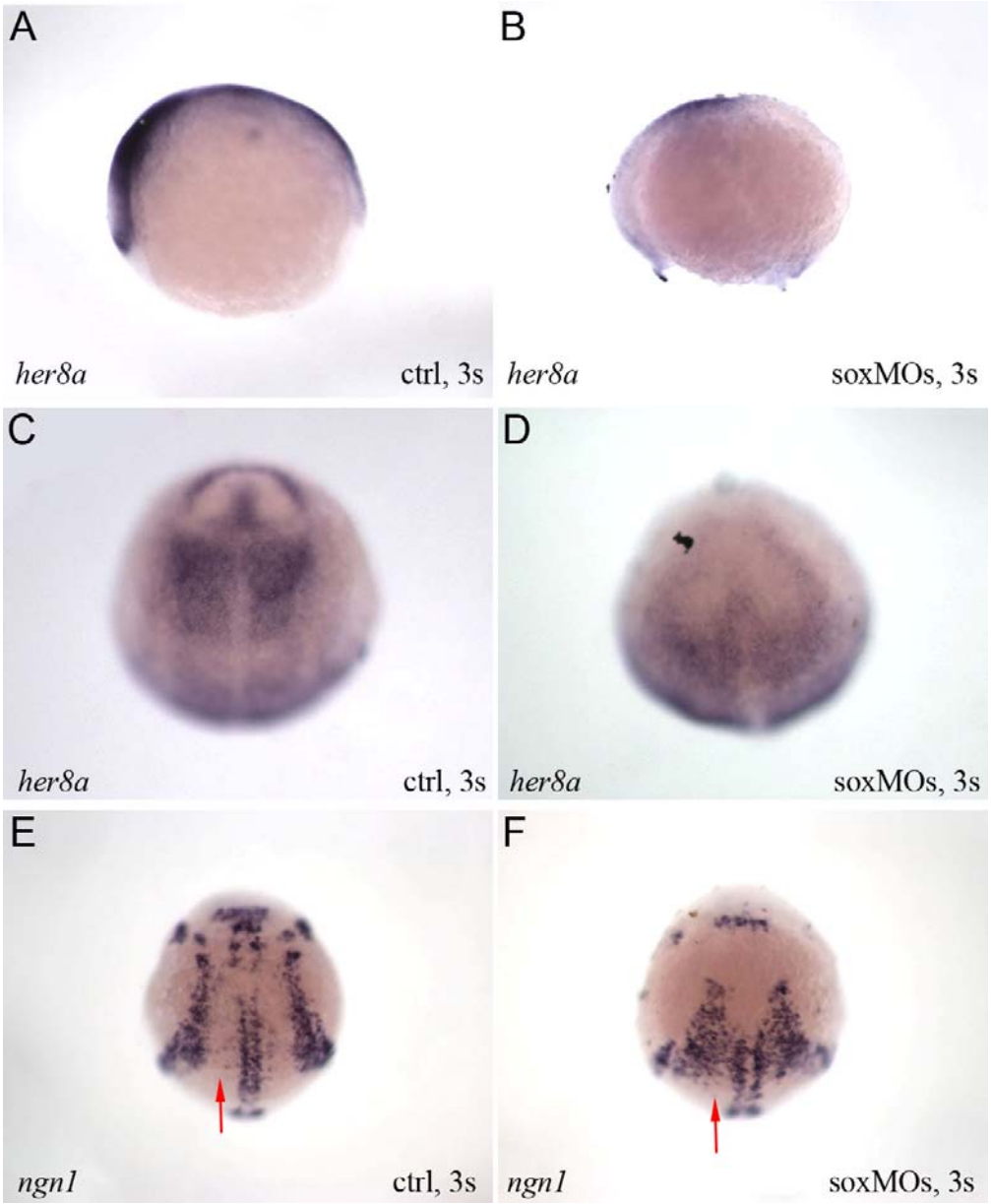

Figure 33. Combined morpholino knockdown of Sox genes reduces *her8a* expression and induces ectopic *neurog1* expression. All expression revealed using in situ hybridisation (blue staining). Embryos were injected with a mixture of morpholinos designed to knockdown *sox2/3*, *sox19a*, *sox19b* and *sox21b*. These embryos were consequently fixed and stained for *her8a* (B, D), or *neurog1* (F) and compared with non-injected controls (A, C, E). *her8a* expression was reduced upon morpholino injection throughout the embryo, whereas ectopic *neurog1* expression was observed, which was restricted to the posterior neural plate (see red arrows).

Table 7. Injection counts for morpholinos against *her8a* and *sox*-family members.

Material injected	Stained for/stage examined	Phenotype (compared to the appropriately treated control)	Proportion showing phenotype (percentage)	Proportion delayed (did not reach stage of analysis) (percentage)	n
her8a MO e1 (1mM)	*sox19a*	No phenotype	-	2/9 (22.2%)	9
her8a MO e1 (1mM)	*sox19b*	No phenotype	-	4/9 (44.4%)	9
her8a MO e1 (1mM)	*sox21*	No phenotype	-	2/11 (18.2%)	11
her8a MO e1 (1mM)	*sox2*	No phenotype	-	1/9 (11.1%)	9
her8a MO e1 (1mM)	*sox3*	No phenotype	-	2/9 (22.2%)	9
sox2/3 (1mM), sox19a (1mM), sox19b (0.25mM), sox21b (1mM) all MOs	*neurog1 at 3s*	Ectopic *neurog1* in mesoderm (individually no phenotype)	10/25 (40%)	4/25 (16%)	25
sox19a, sox19b, sox21b, sox2/3 all MOs	*her8a at 3s*	Decrease in *her8a* expression	18/20 (90%)	2/20 (10%)	20

3.2 *her* gene expression in the zebrafish adult brain

In the next section I will focus on the expression of *her8a* in the adult brain. I choose to focus on three well characterized domains of the adult zebrafish brain: the telencephalon, the periventrcular zone of the hypothalamus and the midbrain. In addition, I compared *her8a*'s expression with other members of the Hairy/Enhancer of split family, namely *her9*, *her3* and *her4*. In the embryo, these genes are expressed in unique, but also in overlapping fashions. *her3* and *her9* are expressed in interproneural domains (Bae et al., 2005). *her4* is expressed alternatively with *neurog1* in proneural domains (Takke et al., 1999). Here I asked the question whether the expression of these genes could give us a clue as to their functions in the adult and whether the different territories of gene expression in the embryo are reflected in the adult.

her8a is expressed at the midline of the adult telencephalon (Fig. 34). Its expression overlaps with a zone of proliferation, which extends across the midline of the telencephalon (Adolf et al., 2006). The expression of *her8a* overlaps with the expression of *her9* (Fig. 34, K-L). The expression of *her9* is broad down the midline, whereas *her8a* expression is thinner and at places restricted to individual cells (blue arrows). The expression of *her8a* closely resembles that of *her4* in the midline of the telencephalon. However, the expression of *her4* extends to

the dorsal surface of the telencephalon, whereas that of *her8a* does not (Fig. 34, M (red arrows)). Proliferating cells are also found at this dorsal surface (Adolf et al., 2006).

her8a is expressed in some ventricular zones of the midbrain (Fig. 35, B-D). *her4*, *her9* and *her8a* are expressed surrounding the ventricle formed by the TSc, Val and TeO on various edges. *her9* is expressed in all three structures forming this ventricle. *her4* is expressed only in the TSc. *her8a* is only expressed at the TSc and TeO border, but not in the Val. The midbrain area contains several zones of proliferation, including the isthmic proliferation zone (IPZ) and the tectal proliferation zone (TPZ) (shown in green in Fig. 35, K). These areas are devoid of *her8a* expression (Fig. 35 B-C, red arrows). *her3* expression can solely be found in the midbrain, at the border between the Val and tegmentum (Teg) and Val and TSc.

her8a is expressed in the dorsal zone of the periventricular hypothalamus (Hd) (Fig. 36, A-G). *her9* and *her4* are also expressed at the Hd (Fig. 36, B-G). In addition, *her9* and *her4* are expressed in the caudal zone of the periventricular hypothalamus (Hc), an area devoid of *her8a* staining. *her3* is not expressed in the hypothalamus.

In summary, *her8a* is expressed in discrete areas of the ventricular zone in the zebrafish adult brain. Its expression overlaps with that of *her3*, *her4* and *her9* at places. *her3* has very limited expression in the adult brain, and it always shares its expression domains with *her8a*. There are many areas where *her9* and *her4* are expressed in exclusion of *her8a*. *her8a* is expressed in areas of proliferation, such as the midline of the telencephalon, however it remains unclear whether *her8a* is expressed in the proliferating cells themselves. Indeed, *her4* expression is excluded from cells expressing the proliferation marker PCNA (P. Chapouton, personal communication). However, it is not expressed in two areas of proliferation of the midbrain – the IPZ and TPZ – and on the dorsal surface of the telencephalon.

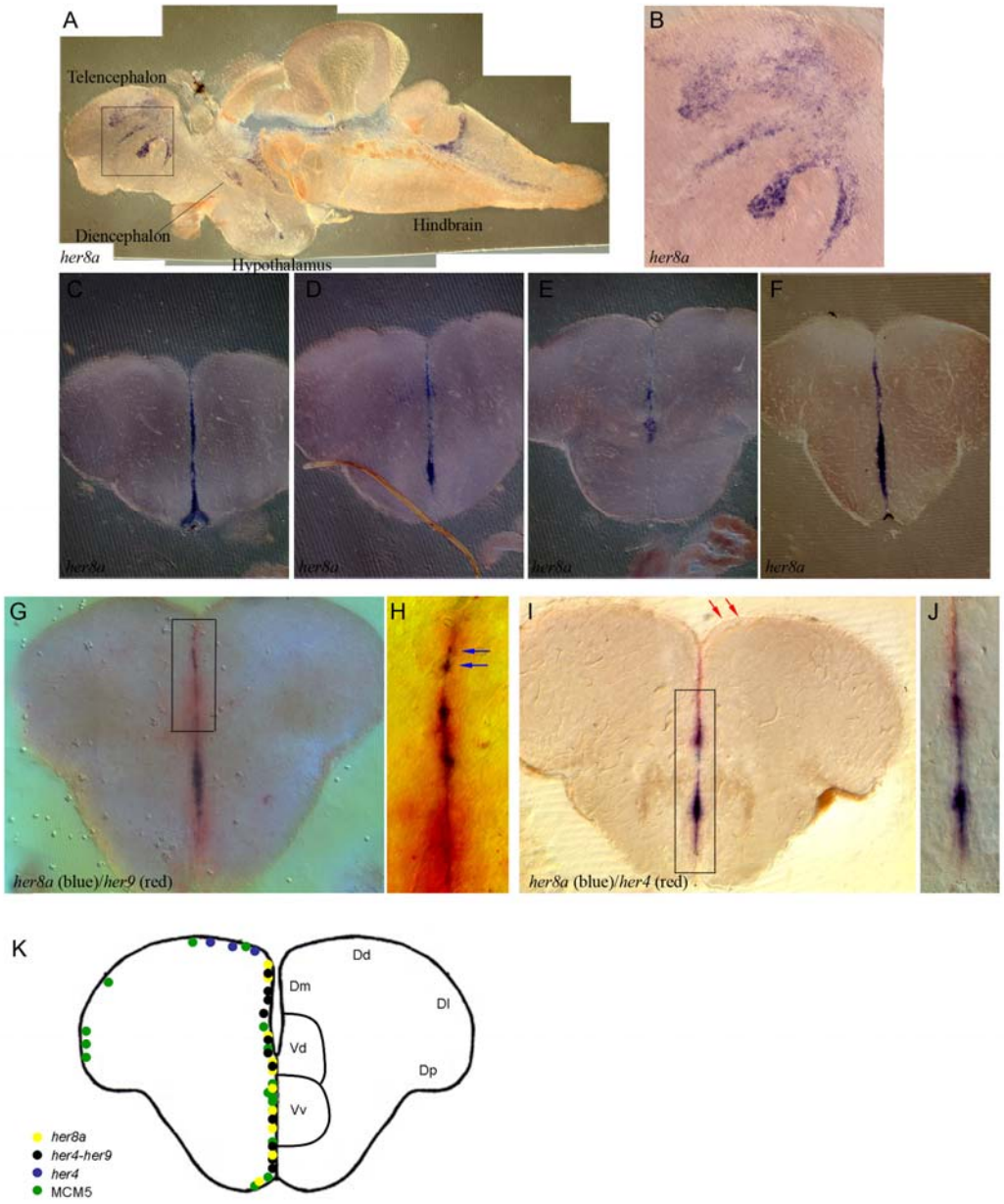

Figure 34. Expression of *her8a* in the zebrafish adult telencephalon. All expression is revealed by in situ hybridization (blue and red stainings). Rectangles outline higher magnifications of the same or equivalent sections. *her8a* is expressed in the midline of the adult telencephalon (A, mid-saggital section; B close-up of A indicated by rectangle); C-J (cross sections, shown from anterior to posterior). *her9* is also expressed in the midline, in a broader fashion than *her8a* (G) and close-up in H. *her8a* can be seen in individual cells (blue arrows). *her4* is expressed similarly to *her8a* in the midline of the telencephalon (I) and close-up in J. All of the *her* genes analysed show discrete but overlapping patterns of expression as depicted schematically in K. *her4/her8a* double stainings courtesy of P. Chapouton.

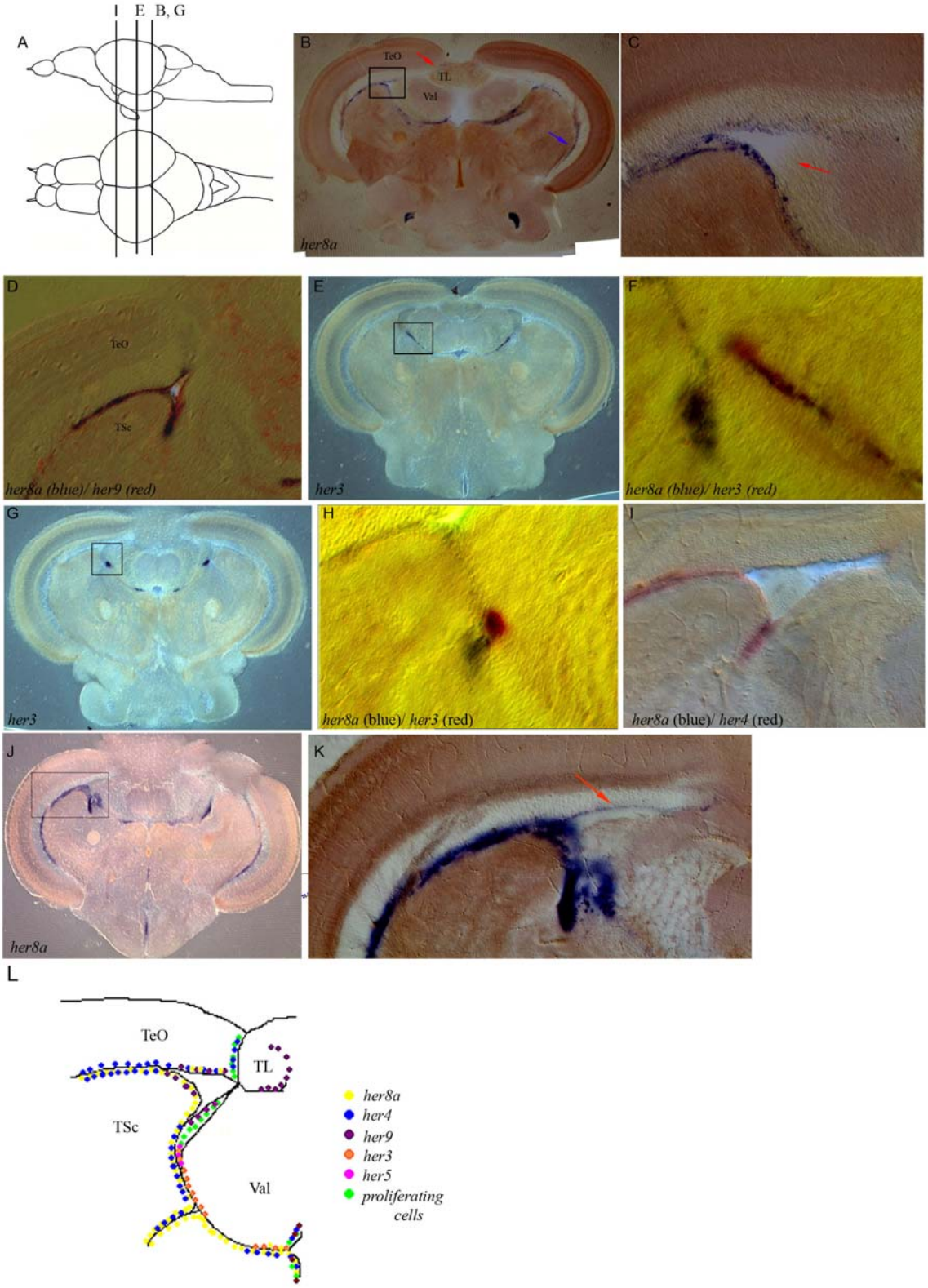
I E B, G
A
B
TeO
TL
Val
her8a
C
D
TeO
TSc
her8a (blue)/ her9 (red)
E
her3
F
her8a (blue)/ her3 (red)
G
her3
H
her8a (blue)/ her3 (red)
I
her8a (blue)/ her4 (red)
J
her8a
K
L
TeO
TL
TSc
Val
her8a
her4
her9
her3
her5
proliferating cells

Figure 35. Expression of *her8a* in the zebrafish adult midbrain. All expression is revealed by in situ hybridization (blue and red staining). Rectangles outline higher magnifications of the same or equivalent sections. *her8a* is expressed in the ventricular zone of the tegmentum and at the border of the periventricular gray zone of the tectum opticum (TeO) (blue arrow). *her8a* expression surrounds the ventricle at the torus semi-circularis (TSc), valvula cerebelli lateralis (Val) and TeO, at the TSc and TeO edges (C-D). *her9* expression completely surrounds this ventricle (D). *her8a* is not expressed at the border between the TeO and TL and at Val border of the ventricle (red arrows in B and C). *her3* is expressed solely in the midbrain, at the border between the Val and Teg and Val and TSc. The expression of *her3* overlaps with that of *her8a* (F and H). *her4* is expressed at the TeO and TSc edge (I). At higher magnification, a thin line of *her8a* positive cells extends over border of the tectum (J, close-up in K, red arrow). All of the *hairy/Enhancer of split* genes studied here have individual and unique expression patterns that frequently overlap (L). Dots indicate areas of expression only and do not indicate that these genes are expressed singularly. *her4/her8a* double stainings courtesy of P. Chapouton. TeO, tectum opticum; TL, torus longitudinalis; TSc, torus semi-circularis; Val, valvula cerebelli lateralis.

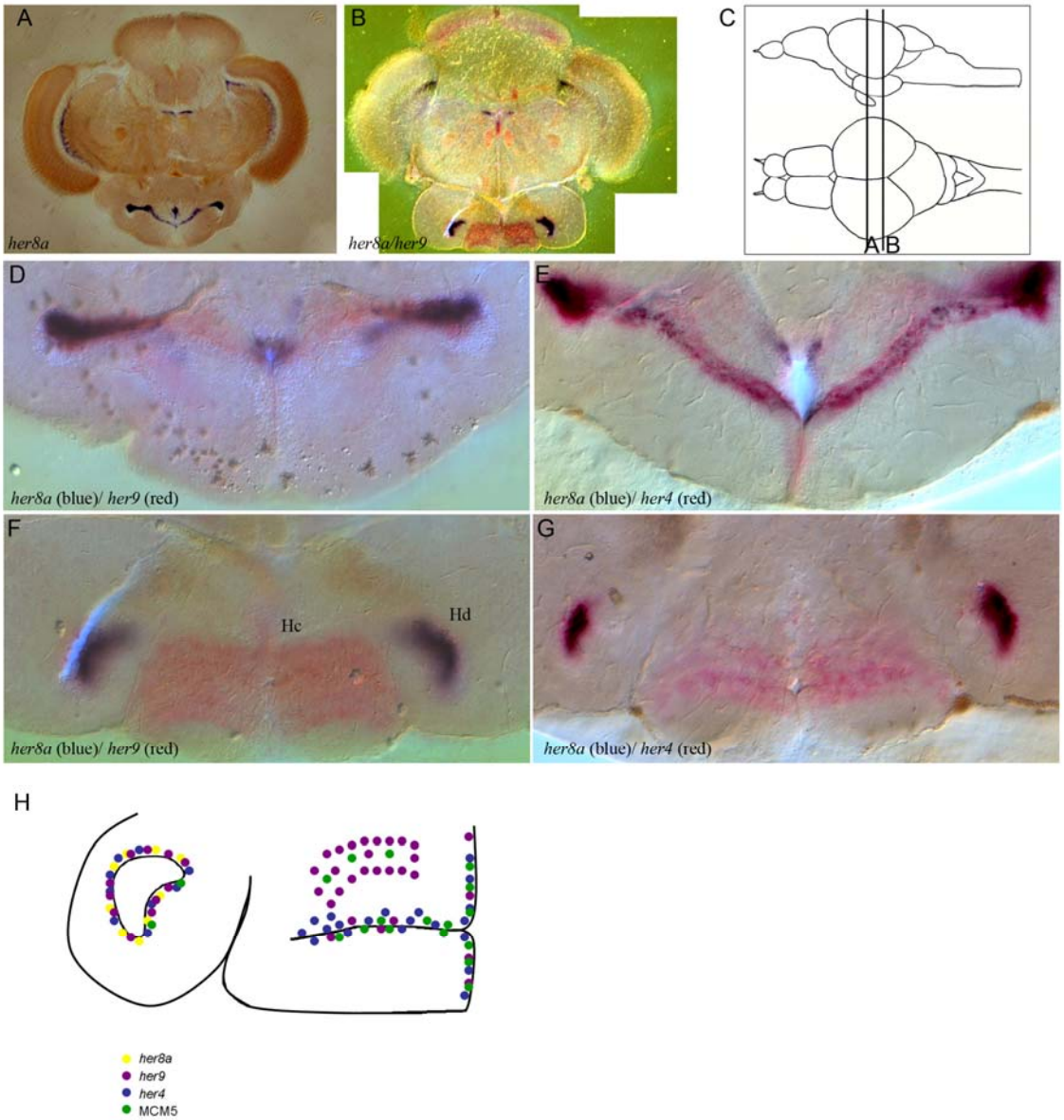

Figure 36. Expression of *her8a* in the zebrafish adult hypothalamus. All expression is revealed using in situ hybridisation (blue and red staining). Expression is shown in a more anterior (A) and posterior (B) plane, as indicated in C. *her8a* is expressed in the dorsal zone of the periventricular hypothalamus (Hd), along with *her4* and *her9* (A, B, D-G). *her4* and *her9* are expressed at the central nucleus of the ventral hypothalamus (Hc) (F-G). This area is devoid of *her8a* staining. The expression of the examined *her* genes occurs in discrete but overlapping areas as depicted schematically in H. Hd, dorsal zone of the periventricular hypothalamus; Hc, central nucleus of the ventral hypothalamus. *her4*/*her8a* double stainings courtesy of P. Chapouton.

3.3 The molecular characterisation of the *no addiction (nad)* mutant

One of the aims of my thesis was the molecular characterisation of *nad*, a mutant previously isolated in our laboratory and that fails to respond to amphetamine. To this end, I used the conditioned place preference set-up previously described in Ninkovic and Bally-Cuif (2006), in order to isolate 36 mutant and 36 wildtype fish. Genomic DNA from tail clips was prepared from these fish and the mutation is currently being mapped in collaboration with Dr. Robert Geisler (Max Planck Institute for Developmental Biology, Tübingen).

3.3.1 Microarray experiments

Parallel to this approach, I took the brains of these identified fish and extracted total RNA in order to use them for microarray analysis. Three microarray experiments were performed in total. In a first comparison, newly identified mutants administered with amphetamine 30 minutes before death were compared with their wildtype siblings. Then in a second comparison mutants without amphetamine were compared with siblings without amphetamine. The genes isolated in this second experiment were removed from those from the first in order to remove the basal transcriptional differences between the mutants and wildtypes. In a third experiment wildtype fish administered with amphetamine were compared with wildtype fish administered with a saline control. The overlap was then taken between the first (minus the basal differences) and the third experiment, in order to isolate genes that are part of the wildtype response, but at the same time responding abnormally in the mutant. We named this set of genes our 'reward pool' (see figure 37).

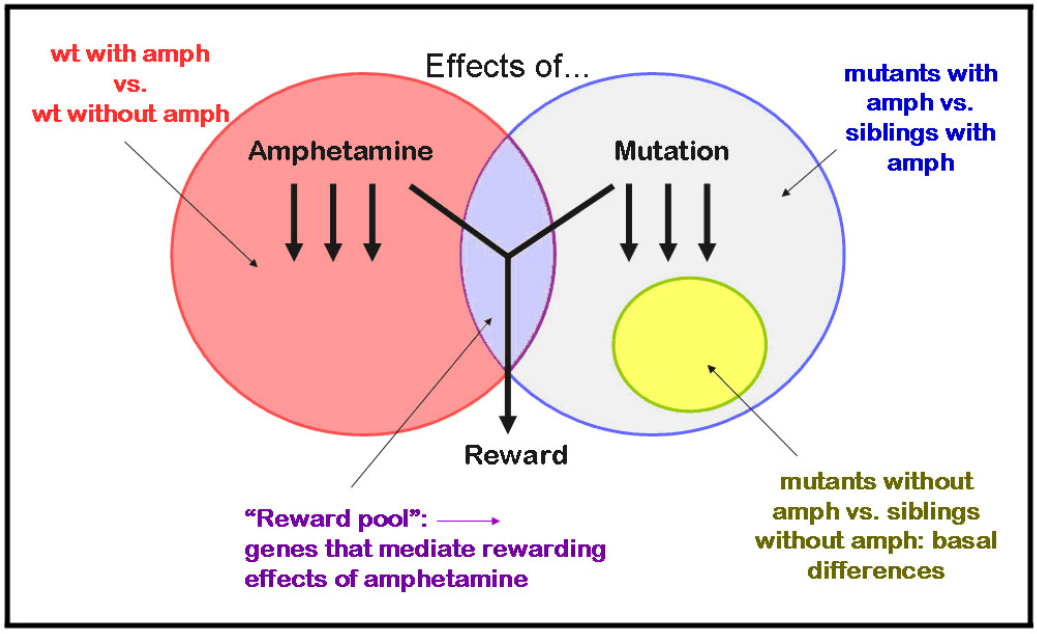

Figure 37. Three microarray comparisons were combined to isolate the 'reward pool'.

GO enrichment analysis shows that there is a comparative enrichment of transcription factors in this reward pool over the complete transcriptome of zebrafish, as well as over the individual experiments. In addition, ten of these candidates were cloned and shown to be expressed in areas of proliferation in the adult brain. Seven genes could be validated using qPCR and four of these could also be validated using in situ hybridisation. qPCR was also used to show that this experiment identified geens responding to the chronic administration of amphetamine.

The results of this work have been compiled in the manuscript in Appendix 8.3.

3.3.2 zfishDB

Network analysis is a way of linking proteins based on information about function or phylogeny. First of all I tried to analyse our microarray results using commercially available software. This failed to produce meaningful linkage between proteins. Network analyse of this kind largely relies on information from published abstracts. Although the amount of published work on zebrafish is growing, there are still comparatively less abstracts available than say on mouse. As a solution to this problem, I collaborated with the 'computational modelling in biology' group headed by Dr. Fabian Theis of the Helmholtz Zentrum München. We developed a database that translates the zebrafish proteins into their mouse orthologues before performing network analysis. This allows not only zebrafish, but also mouse, data to be used to functionally link the proteins, leading to many more links. This database can be accessed under: http://mips.gsf.de/zfishdb/. The resulting network analysis of the reward pool is shown in the manuscript in Appendix 8.3.

3.4 Amphetamine causes premature maturation of progenitor cells in the adult brain

Our analysis of the expression patterns of selected 'reward pool' candidates revealed that they were expressed in areas of proliferation in the adult brain. Most strikingly, *her15* and *gfi1b*, which appear to be completely down-regulated upon amphetamine administration, are expressed directly in the ventriclar zones of the adult brain (see Appendix 8.3), areas known for proliferation and neurogenesis (Adolf et al., 2006; Grandel et al., 2006). Investigations on the effect of amphetamine on neural progenitor proliferation in the rodent adult brain have been inconclusive, likely due to varying administration schedules (Eisch and Harburg, 2006). This led us to investigate the effect of amphetamine administration on the cell cycle and fate of progenitor cells in the adult zebrafish brain. I performed a series of experiments comparing the characteristics of progenitor cells in brains of fish injected with amphetamine vs. those injected with a saline control. I followed an injection scheme over 18 days, from which fish were removed at specific intervals for analysis (see figure 38). The brains were sectioned and stained with antibodies against MCM5 (labels proliferating cells), BrdU (labels cells that have incorporated BrdU during division) and Hu (labels newly committed postmitotic neuronal cells as well as mature neurons, making it an early marker for neuronal differentiation (Mueller and Wullimann, 2002)). I chose the dorsal subpalium of the telencephalon, an area

containing a good representation of ventricular cell types (P. Chapouton, unpublished data), as opposed to the ventral subpalium, which is biased towards fast proliferating cells (Adolf et al., 2006). Care was taken to analyse equivalent sections. As a result only one section was used per brain.

As amphetamine administration decreases *her15* and *gfi1* expression in an area of proliferation, we first investigated whether the drug affects the number of proliferating cells. In a first experiment fish were injected with amphetamine once a day for eight days. The fish were sacrificed 30 minutes after the last injection and the brains were sectioned and stained for MCM5. There is a trend towards less MCM5 labelled cells in the brains of fish injected with amphetamine (see Fig. 39, A, and table 8).

It is possible that a decrease in the number of proliferating cells is caused by non-specific cell death caused by amphetamine administration. Therefore, we stained the sections with cleaved caspase, which is responsible for the proteolytic cleavage of many key proteins, and is thus a marker for apoptosis. We noticed no increase in the amount of cell death in the brains of amphetamine treated fish (Fisher's exact test for the comparison of the proportion of 0 counts (3 out of 4 vs. 2 out of 4) p-value=1; 95%-confidence intervals [0.301,0.987] and [0.150,0.850]) (see also Fig. 39, B).

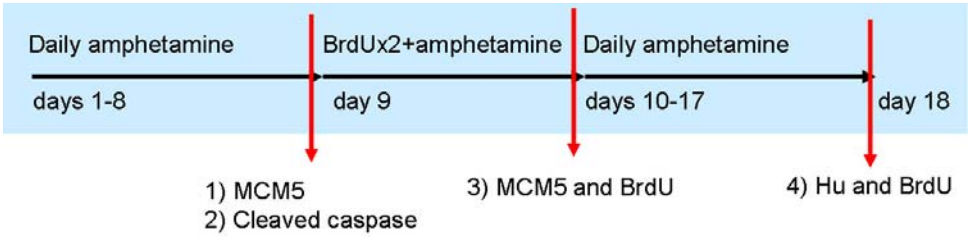

Figure 38. Injection scheme for fish treated with amphetamine for cell counting experiments. Fish were injected with amphetamine once daily for eight days. On day nine fish were injected twice with BrdU, two hours apart, and then once with amphetamine, 30 minutes after the last BrdU injection. Then the fish were treated with amphetamine for a further eight days. On day 18 the fish were given the daily dose of amphetamine and sacrificed 30 minutes later. The control fish were injected with saline solution instead of amphetamine, in the same schedule. At various points (indicated by the red arrows) fish were removed from the injection scheme and sacrificed for analysis, 30 minutes after the daily amphetamine injection. The brains were then sectioned and stained as indicated. The stainings investigate the effect of amphetamine on 1) cell proliferation, 2) cell death, 3) cell cycle speed and 4) differentiation into neurons.

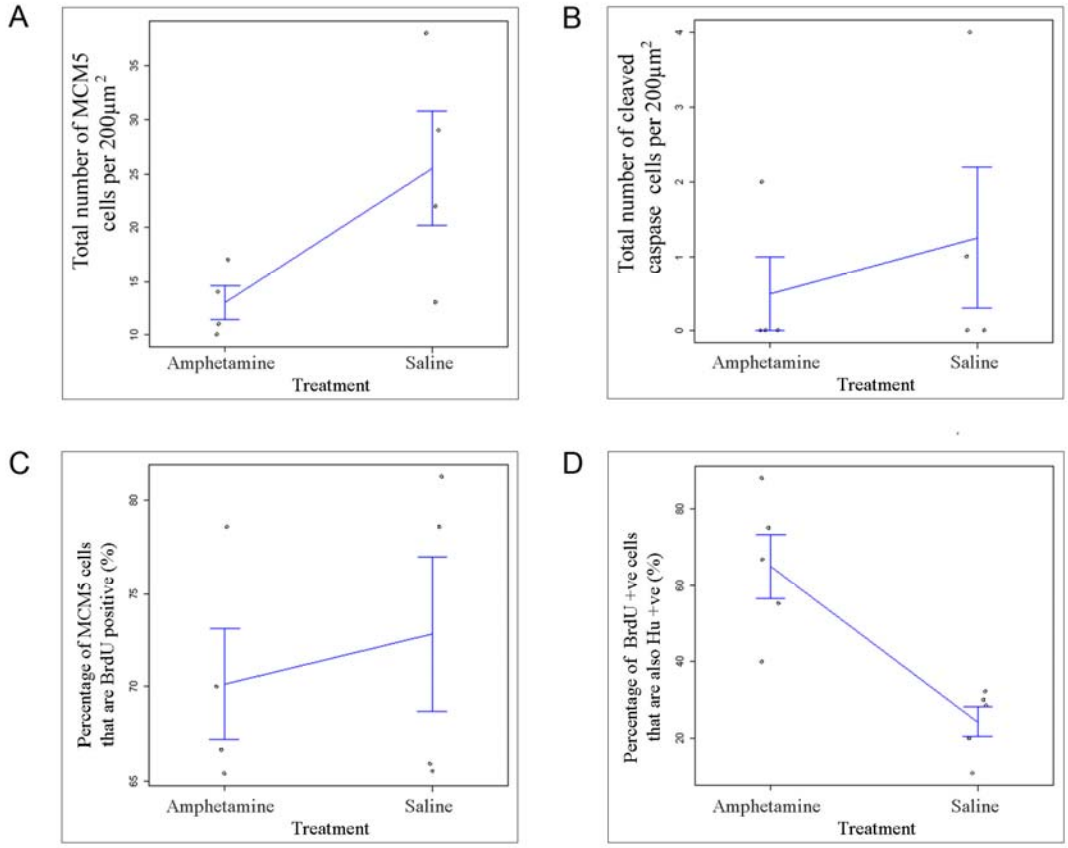

Figure 39. Amphetamine administration can cause premature neuronal differentiation and a tendency towards a decrease in the number of proliferating cells. The brains of fish injected with amphetamine, once daily over eight days, show a tendency towards less MCM5 cells (A). This loss of MCM5 cells can not be explained by increased cell death (B). The cell cycle speed also appeared to be unchanged (C). The number of BrdU cells that had turned on Hu expression as of eight days was increased in the amphetamine injected fish (D). Error bars show the 1 fold of the standard error of the mean.

It is also possible that the cell cycle speed is affected upon amphetamine administration. Sacrificing the fish shortly after a pulse of BrdU allows us to examine the proliferation speed, by calculating the labelling index; that is the proportion of cells in S phase within the whole cycling population. We injected fish once per day with amphetamine for eight days. On day nine we injected the fish twice with BrdU with an interval of two hours. The brains were then sectioned and stained for MCM5 and BrdU. We then counted the number of cells that were MCM5- and BrdU-positive vs. the total number of MCM5 cells. We observed no significant difference in the percentage of MCM5 cells that were BrdU-positive between the amphetamine and control fish (see Fig. 39, C and table 8). Thus, it appears that the cells were proliferating at a similar rate.

Another possibility for the decrease in proliferating cells in amphetamine-treated fish is that they are differentiating prematurely. In BrdU tracing experiments, 52% of labelled cells from the telencephalon had differentiated into neurons after a period of three days (Adolf et al.,

2006). Sacrifice of animals eight days after a BrdU pulse allows the detection of cells that have differentiated into neurons since the pulse. In a last experiment, we treated the fish with amphetamine once a day for eight days. On day nine I injected the fish twice with BrdU and then once with amphetamine. The fish were then treated with amphetamine for a further eight days. On day 18 the fish were then injected with the daily dose of amphetamine, before being sacrificed 30 minutes later. The percentage of BrdU cells that were Hu-positive – that is, the percentage of cells that were cycling at or after day nine, which subsequently differentiated into neurons during the following nine days – was higher in the amphetamine treated fish (see Fig. 39, D and table 8). From this, it appears that amphetamine administration can push cycling cells into premature differentiation, causing a depletion of the progenitor pool.

Table 8. t-Tests with unequal variances were performed on the cell counting experiments 1) cell proliferation, 3) cell cycle and 4) differentiation. The number of cleaved caspase cells per section was too low (between 0-4 per section) so that no t-Test could be performed. In this case I performed a Fisher's exact test for the comparison of the proportion of 0 counts (see text).

Experiment	Amphet-amine mean	Amphet-amine s.e.	Saline control mean	Saline control s.e.	t-Test with unequal variances (p-value)
1) cell proliferation: total number of MCM5 labeled cells after 8 days amphetamine	13.0	1.58	25.5	5.30	0.096
3) cell cycle speed: percentage of MCM5 cells that are BrdU positive	70.156%	2.969	72.812 %	4.136	0.62
4) differentiation: percentage of BrdU cells that are Hu positive	64.99%	8.22	24.39%	3.91	0.0048

4. Discussion & Perspectives

4.1 *her8a* is a repressor of the proneural gene *neurog1*

her8a is a novel member of the bHLH family of transcription factors. I have established its role as a negative regulator of neurogenesis using two lines of evidence. Firstly, I showed that morpholino-mediated knockdown of *her8a* causes ectopic *neurog1* expression in rhombomeres 2 and 4. Secondly, the overexpression of full-length *her8a* causes a complete loss of *neurog1* expression in the early embryo. I have shown that *her3* and *her8a* play an equally important role in the maintenance of progenitors in rhombomeres 2 and 4 (r2 and r4).

In a similar manner, *her5* and *her11*/*him* function redundantly to maintain the lateral part of the progenitor zone at the midbrain-hindbrain boundary (mhb) (Geling et al., 2003; Geling et al., 2004; Ninkovic et al., 2005). *her5* and *her11*/*him* are found back-to-back and close together on the same chromosome. It is likely that they share the same regulatory elements as the enhancer activity driving the expression of *her5* at the mhb extends into the *her11*/*him* locus (Tallafuss and Bally-Cuif, 2003). However, unlike *her5* and *her11*/*him*, *her3* and *her8a* do not perform redundant functions in a dose dependent manner and, as they are positioned on separate chromosomes, they can not share regulatory elements. If *her8a* and *her3* morpholinos are injected together at concentrations just below that needed to cause a phenotype, ectopic *neurog1* expression is not observed. In addition, the injection of *her8a* and *her3* morpholinos at active doses does not produce an additional phenotype. Thus, they are not equally responsible for progenitor maintenance in r2 and r4 in a dose dependent manner.

Progenitor maintenance at the mhb of the mouse also relies on the redundant actions of Hes1 and Hes3 (Hirata et al., 2001). Like Her3 and Her8a, the genes encoding these factors are not genetically linked and their expression patterns are distinct from each other, only overlapping at the mhb (Allen and Lobe, 1999; Lobe, 1997).

her8a is expressed broadly throughout the embryo at early segmentation stages. Therefore, it is surprising that its morpholino phenotype is restricted to rhombomeres 2 and 4. It is possible that under normal conditions *her8a* only restricts *neurog1* expression in these zones. This is unlikely, given the broad expression pattern of *her8a*. It is also possible that *her8a* is working redundantly with other proteins in other regions, and that its absence can be compensated for in the majority of progenitor zones in the embryo. The *neurog1* phenotype of caused by *her8a* or *her3* knockdown is at the area of overlap of *her8a*/*her3* expression, in an area devoid of *her5* (see figure 40). It is possible that *her8a* can only act to inhibit *neurog1* in areas of dense expression, and that *her5* and *her11*/*him* can compensate for its loss at the mhb progenitor pool. Functional redundancy is common between members of the *E(spl)* family. In *Drosophila*, six out of the seven *E(spl)-C* genes exhibit identical expression patterns in the neuroectoderm (Knust et al., 1987; Knust et al., 1992). In zebrafish, *her4* and *her2* are expressed identically in the neural plate (Takke et al., 1999) and *her5* and *her11*/*him* are expressed identically at the presumptive mhb (Ninkovic et al., 2005).

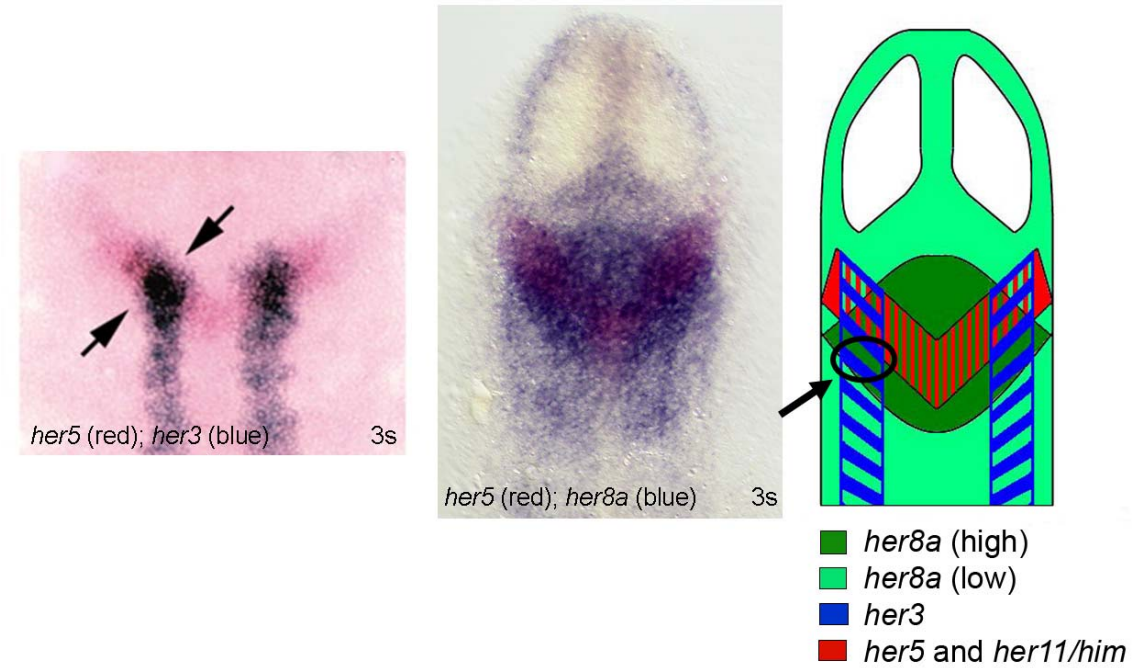

Figure 40. Position of knockdown phenotype with regard to expression. The area of the phenotypes of *her3* and *her8a* knockdown is at the area of overlap between the area of dense *her8a* expression and *her3* expression, but not in areas of *her5* or *her11/him* expression.

In order to ascertain if the loss of one protein can be compensated for by another, it is possible to knockdown the expression of one gene, while overexpressing the other. Due to technical difficulties (the reaching of a combined toxic dose), I could not overexpress *her8a* at the same time as *her3* morpholino. In addition, I was unable to reproduce the published *her3* misexpression phenotype (the loss of *neurod4*) , despite using the construct from the original publication.

4.2 *her8a* is expressed in a broad manner at early embryonic stages that gradually becomes more restrictive

her8a is unusual in that it is expressed in proneural clusters, as well as in progenitor pools. Other members of the *her* family that are expressed in proneural clusters are missing from progenitor zones (e.g. *her2*, *her4*, *her12* and *her15* (formerly *hes5*) (Takke et al., 1999)). *her8a* appears to be responsible for the maintenance of progenitors, in a similar manner to *her3*, *her5*, *her9* and *her11/him*. The expression of these genes is restricted to progenitor pools and it does not overlap with that of *neurog1* (Bae et al., 2005; Geling et al., 2003; Ninkovic et al., 2005). In this work I showed that the misexpression of *her8a* can inhibit *neurog1* expression. It is possible that this inhibition occurs in a dose-dependent manner, only occurring at non-physiological doses in proneural clusters. It is also possible that *her8a*

is expressed alternatively with *neurog1*. Indeed, denser stripes of expression can be seen at 10 somites in the anterior of rhombomere 1, an area free of *neurog1* expression at this stage.

It is possible that the less-dense expression represents alternative expression in a salt-and-pepper manner, with the differences too subtle to be observed using in situ hybridisation. Conversely, it could be that in the areas of high expression, *her8a* is expressed at high levels and continuously in every cell. If this is the case, it would have parallels to the expression of *Hes1* in the mouse. *Hes1* is expressed in neurogenic zones alternatively with cell expressing proneual genes, such as Mash1 (Baek et al., 2006). In contrast, at boundaries, such as at the mhb, *Hes1* is expressed at high levels in all cells.

In the neurogenic zones *Hes1* responds to lateral inhibition through Notch signalling. *her8a* does not require Notch for its expression throughout the embryo at early segmentation stages, indicating that this is not a direct parallel situation. However, the onset of expression of *Hes1* in the mouse neural plate precedes Notch expression (Hatakeyama and Kageyama, 2006), so *Hes1* can also not be dependent on Notch signalling at early stages. These studies suggest that members of the Hairy and Enhancer of Split family can be regulated by different mechanisms, according to their time and place of expression in the embryo and adult. This study adds to earlier work showing that the response of *her* genes to Notch depends on their cellular context. Further study is needed to show how this is achieved at the molecular level.

4.3 Her8a is most closely related to mouse Hes6 and it has an intermediate loop length

Her8a closest homologue in mouse is Hes6. Mouse *Hes6* inhibits *Hes1*, and thus is a positive regulator of differentiation (Bae et al., 2000). *Hes6* expression continues in differentiated cells, even after they stop expressing *Hes1* (Bae et al., 2000). Hes6 alone does not bind to N box or E box sequences. However, it can interfere with the E box binding of Hes1. This is similar to the HLH factor Id1, a dominant negative regulator of bHLH factors (Benezra et al., 1990). Since Id1 lacks the basic region, it cannot bind DNA by itself. However, it forms heterodimers with other bHLH factors through the HLH domain and interferes with their DNA binding. It has been hypothesized that Hes6 interferes with E box binding of Hes1 in a similar manner (Bae et al., 2000).

Her8a has three less amino acids in its loop than other members of the bHLH family, therefore it has two more than other Hes6 family members (Bae et al., 2000). The addition of five amino acid residues into the loop of Hes6 confers Hes1-like repressor activity on the N box. Conversely, the removal of five amino acid residues from the loop of Hes1 completely ceases repression activity and confers Hes6-like activity (Bae et al., 2000). Therefore, the loop region is necessary for the proper functions of Hes6 and Hes1. Her8a has an intermediate number of amino acids. The functional significance of this intermediate loop-length remains a matter for investigation.

Several lines of evidence suggest that the function of *her8a* has diverged from that of mammalian and *Xenopus* Hes6 (Koyano-Nakagawa et al., 2000). Hes6 expression in the developing nervous system in both mouse and Xenopus correlates with neurogenesis. In both

species the expression of Hes6 starts after that of the neurogenins, but precedes that of differentiation genes. The expression of *her8a* is not restricted to proneural genes, and it does not correlate with zones of neurogenesis. In addition *Xenopus Hes6* is not sensitive to Notch intracellular domain expression, in constrast to *her8a*, which responses with upregulation in most areas. Lastly, the ectopic expression of *Hes6* in *Xenopus* embryos promotes neurogenesis, whereas this work establishes *her8a* as a negative regulator of neurogenesis.

In addition to Her8a, there are two other Hes6 homologues in zebrafish – Her13 (formerly Her13.1) and Hes6 (formerly Her13.2). Both of these factors were found to interact with Her5 in the yeast-2-hybrid screen. I cloned both of these genes and analysed their expression patterns. *her8a*, *her13.1* and *her13.2* have very contrasting expression patterns. On the basis of the divergent expression patterns, and the differences to Hes6 mentioned above, it seems reasonable to suspect that only one or a combination of these genes share the original function, leaving the others to diverge in function. Investigations into the functions of Her13 and Hes6 would help elucidate the functional relationship between Hes6 in other species and Hes6 homologues in zebrafish.

4.4 Factors working upstream of Her8a

4.4.1 Regulation by other Her members

her8a knockdown did not change the expression pattern of *her3*. In addition, combined knockdown of *her3*/*her5*/*her9*/*her11*(*him*) did not affect *her8a* function. Therefore it appears that they are not working upstream of each other. Hes6 has been shown to play a role in the inhibition of Hes1 activity, by forming a heterodimer with Hes1 (Bae et al., 2000). I am currently performing co-immunoprecipitation experiments, which I hope will give an insight into the mode of function of Her8a. y2h using Her5 as bait was originally used to recover binding partners of Her5. However, *her8a* knockdown produces the same phenotype as *her3* knockdown, so it is possible that Her8a and Her3 also interact. In addition, y2h is not a precise method of determing interaction parters. Therefore, I want to see whether Her3 binds with Her8a and whether Her3 and Her8a can form homodimers with themselves (see figure 41).

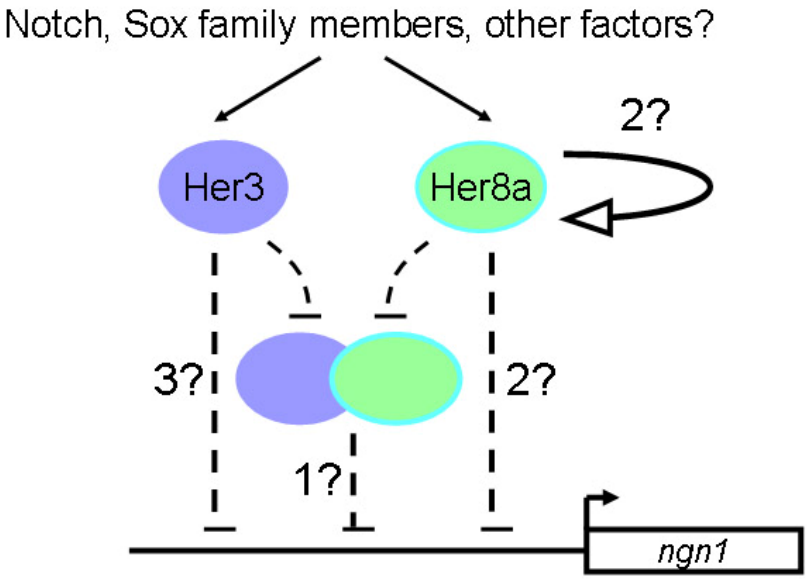

Figure 41. Co-immunoprecipitation will be used to help establish the mode of action of Her8a and Her3. If Her3 and Her8a are able to bind the promoter of *neurog1* directly, it is possible that they could do it in the following combinations: 1) Her3-Her8a, 2) Her8a-Her8a and 3) Her3-Her3.

4.4.2 Autoregulaton of *her8a*

Morpholino knockdown of *her8a* causes a complete loss of *her8a* expression. This indicates that *her8a* activates its own transcription. In contrast, *her3* knockdown increases the density of *her3* transcripts in all of the *her3* expression domains, suggesting that Her3 represses transcription of its own gene within the limits of its expression (Hans et al., 2004).

4.4.3 Notch signalling

Many members of the *E(Spl)* family have been shown to be activated by Notch signalling, both in *Drosophila* (Bailey and Posakony, 1995; Jennings et al., 1994; Lieber et al., 1993) and vertebrates (Jarriault et al., 1995; Takke and Campos-Ortega, 1999). The blocking of Notch signalling using DAPT treatment does not perturb the expression of *her3*, *her5* or *her9* at early segmentation stages (Bae et al., 2005; Geling et al., 2004). *her5* and *her3* expression is inhibited rather than activated by NICD overexpression (Bae et al., 2005; Geling et al., 2004), whereas *her9* expression remains unchanged (Bae et al., 2005). Thus, it appears that they do not, at least at this moment, require Notch for their expression. *her8a* also does not respond to DAPT at three somites. However, its expression is upregulated by NICD, indicating that its expression can be activated by Notch under non-physiological conditions. This led me to investigate the effect of Notch at later stages. At later stages DAPT treatment causes a complete downregulation of *her8a*, as shown at 48hpf (this thesis) and in the adult brain (P. Chapouton, personal communication). The expression of *her3* and *her9* is partly dependent on Notch signalling at later stages (Bae et al., 2005). Thus it appears that the responsiveness

of *her* genes to Notch is not fixed, but that it changes throughout development. This shows that one must be careful when making parallels between the embryo and adult situation.

4.5 Mode of function of Her8a

Transcriptional activators can be made into repressors through the addition of a strong repressor domain from another protein e.g. engrailed from *Drosophila (Jaynes and O'Farrell, 1991)*. In addition, repressors can be turned into activator by fusing a strong transcriptional activator domain from a protein such as VP16 from the cytomegalovirus. If misexpression of the VP16 fusion produces a phenotype similar to the misexpression of the normal factor, the normal factor must be an activator. In this case the phenotype of the engrailed repressor fusion, in which the normal targets of the transcription factor are repressed, gives an indication of the normal functions of the gene (in zebrafish it should mimic the morpholino phenotype). Conversely, if the misexpression of the engrailed fusion produces a similar phenotype to the misexpression of the normal factor, this factor must act as a repressor.

The results of making fusion proteins in order to determine whether *her8a* acts as an activator or a repressor were inconclusive. Neither *her8a*-VP11x2 construct or *her8a*-Eng construct were able to either inhibit *neurog1* expression or cause ectopic *neurog1* expression. Her3 acts as a transcriptional repressor. Embryos expressing the Her3eng fusion have fewer primary neurons, whereas Her3VP16 shows ectopic *neurog1* transcription. So, *her3*eng RNA causes the same phenotype as *her3* missexpression alone. Using similar mechanisms, *her9* has also been shown to act as a transcriptional repressor (Bae et al., 2005).

At a molecular level, Hairy/E(Spl) factors have several mechanisms to restrict neurogenesis. It is possible that Her8a binds the promoter of *neurog1* directly in order to repress its transcription. Indeed, I showed that a *her8a* construct without a basic domain was unable to inhibit *neurog1* expression. Hans et al., (2004) used gel retardation assays to show that Her3 represses transcription by binding directly to N-boxes, a major DNA target for E(Spl) proteins (Oellers et al., 1994; Sasai et al., 1992; Tietze et al., 1992). Gel retardation assays would show whether Her8a causes *neurog1* repression by binding directly to its promoter.

4.6 *her* genes in the adult

This work and work by other members of this group have investigated and compared the expression of Her factors in the zebrafish brain. It is interesting to ask whether the processes discussed above are involved in progenitor maintenance or in proneural processes in the adult. The adult brain of teleost fish contains many zones of proliferation (Kaslin et al., 2008; Lindsey and Tropepe, 2006). Recent studies in zebrafish have analyzed the nature and fate of proliferating cells. A short pulse of the thymidine analog BrdU, followed by cell tracing, shows that fast proliferating cell populations give rise to neurons throughout the brain (Adolf et al., 2006; Castella et al., 2000; Grandel et al., 2006; Pellegrini et al., 2007; Zupanc et al., 2005). Most proliferating zones overlap with domains expressing proneural genes or the Notch target *her4* (Chapouton, personal communication). This suggests that at this level adult neurogenesis is controlled by factors used in the embryo. Cumulative injections of BrdU, followed by long-term tracing and staining for proliferation markers, such as MCM5 or

PCNA, demonstrate that proliferation zones also contain slow-dividing precursors. These do not dilute the BrdU label and they remain in cycle over long time periods. These long-lasting progenitors express the transcription factors Sox2 and Pax6b (Adolf et al., 2006; Chapouton et al., 2006) and can be considered to be adult neural stem cells.

Our laboratory has demonstrated using a transgenic line with GFP under the control of the promoter elements of *her5* that these slow cycling cells express *her5* (Chapouton et al., 2006). Work in this thesis and from other members of this laboratory (P. Chapouton and C. Stigloher, unpublished) have shown that other *her* genes involved in progenitor maintenance are expressed widely in proliferation zones throughout the adult brain. They have overlapping, but at the same time distinct areas of expression, reminiscent of the situation in the embryo (see figure 42). *her8a* is also shown to be restricted to proliferation zones in the adult. Its expression in the adult is much more restrictive than the broad expression in the embryo, and correlates with a gradual restriction of expression as observed at 24hpf and 48hpf.

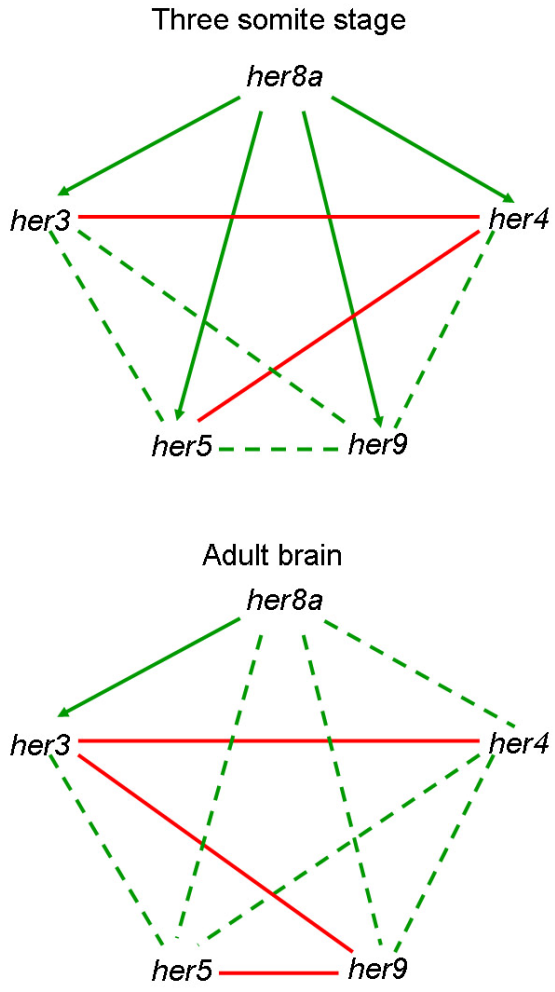

Figure 42. A comparison of the overlap of *her* gene expression in the embryo and in the adult. Green line indicates a complete overlap in expression, red line no overlap in expression and a dotted line some overlap in expression. Where the expression of one gene is broader than that of another, there is an arrow to indicate this (e.g. the expression of *her8a* is broader than that of *her3*).

One important feature will be to see whether the expression of *her* genes in the embryo respond in the same way to Notch in the adult. As part of a joint publication with many

members of our group, P. Chapouton is currently establishing how *her* genes respond to DAPT treatment in the adult live swimming fish. *her8a* expression is down-regulated upon DAPT treatment in the adult, as shown by in situ hybridisation (P. Chapouton, personal communication). If Notch is blocked, *her8a* expression is decreased (P. Chapouton, personal communication). In addition, proliferation is increased and proneural gene expression is increased. This is the opposite as what is happening in the embryo, where loss of *her8a* causes premature differentiation and not increased proliferation. The progenitor cells affected in the adult are quiescent. If you block Notch, these divide before they differentiate into neurons. In contrast the cells in the embryo are proliferating already.

This work shows that *her8a* is expressed in proliferation zones in the adult brain, and that its expression pattern is distinct but at the same time overlapping with other *her* family members. However, in order to gain clues as to the function of *her8a* in the adult brain, a more detailed analysis is required. First of all, it will be interesting to analyze the cellular characteristics of *her8a* positive cells. Despite repeated attempts, we failed to produce double stainings of *her8a* using in situ hybridization, with antibody markers, such as Hu, MCM5 or BLBP. We also could not establish whether *her8a* is expressed in the same cells as the other *her* family members, and if so, whether they are expressed at the same time. In order to investigate this further, we would require antibodies for Her family members or transgenic lines with short-lived marker proteins. No one *her* gene was found to be expressed in all proliferation zones in the adult. It is possible that, like in the embryo, *her* genes are also working redundantly in the adult brain to maintain neurogenesis. Therefore, it appears that there is no master *her* gene regulating adult neurogenesis. However, it is also possible that *her8a* is not playing a role in progenitor maintenance in the adult, but that it is involved in fate specification. In line with this, *Hes6* expression continues in differentiated cells, even after they stop expressing *Hes1* (Bae et al., 2000).

4.7 Isolation of genes linked to reward

I isolated 139 genes linked to both amphetamine administration in the wildtype and to the non-response to amphetamine in *nad* mutants, leading to a first identification of the molecular changes associated with the reward pathway upon administration of amphetamine. This is the first time that transcriptional changes have been isolated that have direct links with reward. Further work would be required to see whether these genes can be related to susceptibility to addiction in humans.

This study only looks at reward with regard to amphetamine administration. It would be interesting to know whether *nad* mutants are also resistant to other addictive drugs, or whether the lack of response is specific to amphetamine. In addition, it would be interesting to know whether other drugs also change the expression of the genes of our reward pool.

The microarray experiments from this thesis do not show changes in immediate early transcription factors, such as Erg2, Krox24, c-fos, c-jun and CREB, which are often transiently up-regulated in response to drug administration (Rhodes and Crabbe, 2005). This, and the qPCR experiment looking at acute and chronic administration, confirms that our gene set reflects rather long lasting transcriptional changes from amphetamine action.

High experimental variation makes it difficult to compare microarray experiments. Many experimental components vary from researcher to researcher. These include animal species, brain regions, methods for RNA extraction and hybridisation, drug treatment and microarray platform. For this reason, it is difficult to compare results from different microarray studies directly. Nevertheless, a general outcome was the response to chronic drug use of molecular pathways controlling neurotransmitter signalling (including receptors, transporters and signal transduction components), ion channels and regulators of neuronal activity and plasticity events such as synaptic function or extracellular matrix remodelling (Lehrmann et al., 2006; Rhodes and Crabbe, 2005; Sokolov et al., 2003; Winstanley et al., 2007; Zhang et al., 2005).

One of the most striking aspects of this work is to highlight the importance of transcription factors with roles in development in the response to amphetamine-triggered reward. In Gene ontology enrichment analysis it was shown that the reward pool contains a further enrichment in transcription factors, above that of the individual experiments and many of these transcription factors have established roles in development. In addition, several of these genes (*Ahr1*, *Dlx1*, *Foxg1*, *Hes5*, *Sox9* and *Tbr1*) have been related to drug use or administration in mammalian studies (Kerns et al., 2005; Ma, 2007; Mayfield et al., 2002; Sokolov et al., 2003; Thibault et al., 2000; Treadwell and Singh, 2004). These observations suggest that the development of CPP behaviour in response to amphetamine, and possibility the development of reward in general, re-uses developmental genes that may possibly retain a modulatory role during adulthood. It is possible that these genes contribute to brain plasticity, which itself is through to contribute to the learning of addictive behaviours and possibly underlie the persistent changes mediating the effects of addiction (Jones and Bonci, 2005). Several recent reports show that embryonic factors can be recycled in the adult to regulate brain plasticity (Sugiyama et al., 2008). It will be interesting to test the function of these factors in the adult brain and to test their importance using behavioural assays.

4.8 The effects of drugs of abuse on adult neurogenesis

Several of the genes that were visibly down-regulated using in situ hybridization are expressed in progenitor zones. This led me to investigate the effect of amphetamine on differentiation and proliferation in the zebrafish adult brain. Cocaine addicts have numerous cognitive deficits that endure even after prolonged abstinence. These comprise of impairments in executive functions, dependent on the prefrontal cortex, as well as deficits on learning and memory tasks sensitive to hippocampal function (Briand et al., 2008). It is tempting to speculate that the changes in adult neurogenesis caused by drugs of abuse contribute to cognitive deficits seen in drug addicts. However, this requires more research and in the meantime the alternative hypothesis that cognitive effects occur through mechanisms other than adult neurogenesis can not be ignored.

My results showed that amphetamine increases differentiation of progenitor cells into neurons in the zebrafish. Immature neurons are important for the function and structure of the hippocampus (Esposito et al., 2005; Markakis and Gage, 1999) and, if these findings can be confirmed in mammals, it is intriguing to consider the impact that this increase in immature neuron number may have on hippocampal function. An increased number of immature neurons may be related to the ability of cocaine to modulate hippocampal influence over

reward circuitry and goal-directed behaviour (Goto and Grace, 2005; Sun and Rebec, 2003) and memory (Kilts et al., 2001).

The functional relevance of cocaine-induced alterations in neurogenesis is some what clearer in the SVZ, as the addition of new SVZ neurons to the olfactory bulb indisputably enhances aspects of olfaction (Gheusi et al., 2000). Cocaine reduces olfactory function in humans, and this phenomenon is observed even if the cocaine is administered intravenously, thus it is not due to nasal septum damage (Podskarbi-Fayette et al., 2005; Stripling and Ellinwood, 1977). The defects in olfaction improve with abstinence (Bauer and Mott, 1996; Gordon et al., 1990).

It will be interesting to test whether manipulations of adult neurogenesis can modify the susceptibility for addictive behaviour. These studies will need techniques to specifically alter adult neurogenesis with minimal side effects. It is also interesting to note that many of the conditions that are comorbid with substance abuse, such as stress, depression and schizophrenia (Brady and Sinha, 2005), show dysregulation in adult neurogenesis (Paizanis et al., 2007).

The mechanisms by which drugs of abuse can affect adult neurogenesis are still unknown. Almost all addictive drugs enhance dopamine levels in the nucleus accumbens (Wise and Bozarth, 1984) . It has been hypothesized that changes in dopamine stimulation can stop or decrease the synthesis or release of mitogenic factors (substances that encourage cell division) from cells in the vicinity of progenitor cells. Alternatively, it is also possible that dopamine works indirectly through glutamatergic transmission. Acute amphetamine or cocaine administration increases glutamate release in the striatum (Wang and McGinty, 1998) and this increase in glutamate might then decrease cell division in the striatum. In contrast to drugs of abuse, dopamine depletion increases progenitor proliferation. The blockage of dopaminergic receptors using the antagonist haloperidol increases dentate granule cell proliferation in the gerbil hippocampus (Dawirs et al., 1998). In the same way, injections of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), known to selectively damage dopaminergic terminals in the dorsal striatum and cell bodies in the substantia nigra, increase proliferation in the striatal and nigral regions of adult mice (Kay and Blum, 2000; Mao et al., 2001).

A key area of research would be to establish the functional significance of these newly-born neurons with regard to drug reinforcement (see also figure 43). The integration of a newly born neuron takes about three weeks – a lot more time than it takes to establish place preference. Therefore, it can not be the integration of new neurons, which is responsible for the defects in place preference in the *nad* mutants. Astrocytes have not been identified in zebrafish and neurons have been shown to be formed from radial glia. It is possible that the premature maturation of neurons caused by the amphetamine could deprive the neurons at the midline of radial glia re-uptake of neurotransmitters or of trophic support. Further analysis of the mutants will also reveal whether they have defects in adult neurogenesis.

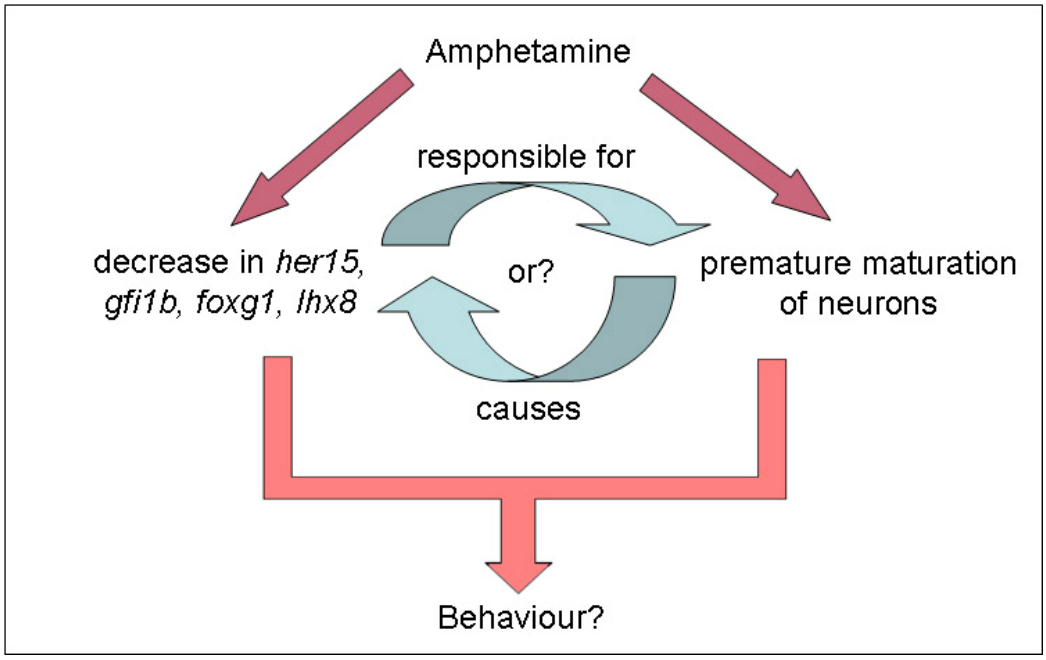

Figure 43. A key area of further research will be to analysis the function of *her15*, *gfi1b*, *foxg1* and *lhx8* at the molecular level. In this thesis I showed that *her15*, *gfi1b*, *foxg1* and *lhx8* are visibly down-regulated in proliferation zones of the adult brain in response to amphetamine administration. It is possible that the decrease in the expression of *her15*, *gfi1b*, *foxg1* and *lhx8* that is caused by amphetamine administration is responsible for the premature maturation of neurons, or it is equally possible that the down-regulation is responsible for this down-regulation. It also remains to be investigated whether one of these observations could affect conditioned place preference behaviour.

We now have the tools in place to investigate the affects of adult neurogenesis deficits in zebrafish on a behavioural level. We can use the conditioned place preference test to measure drug reinforcement under enhanced or decreased neurogenesis using chemicals, and behavioural and cognitive changes induced by the addicted state can be assessed using a variety of tests.

5. Methods

5.1 Yeast Two-Hybrid Analysis

Yeast two-hybrid screening was performed by Hybrigenics, S.A., Paris, France (http://www.hybrigenics.com). The coding sequence for amino acids 20 to 201 of the *Danio rerio* Her5 protein (GenBank proteic accession number gi: 18858797) was PCR-amplified and cloned into pB29 as an N-terminal fusion to LexA (N-Her5-LexA-C). The construct was checked by sequencing the entire insert and used as a bait to screen a random-primed *Danio rerio* embryo (stages 18-20 hpf) cDNA library constructed into pP6. pB29 and pP6 derive from the original pBTM116 (Vojtek and Hollenberg, 1995) and pGADGH (Bartel, 1993) plasmids, respectively.

76 million clones (7.6 -fold the complexity of the library) were screened using a mating approach with Y187 (mat□) and L40□Gal4 (mata) yeast strains as previously described (Fromont-Racine et al., 1997). 280 His+ colonies were selected on a medium lacking tryptophan, leucine and histidine, and supplemented with 2 mM 3-aminotriazole to handle bait autoactivation. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher et al., 2005).

5.2 Alignment and domain analysis

Alignment was performed using ClustalW2, available on the EMBL-EBI website (http://www.ebi.ac.uk/Tools/clustalw2/index.html), using the standard settings. Domain analysis was performed using Prosite (http://www.expasy.ch/prosite/).

5.3 Manipulation of embryos

5.3.1 Zebrafish strains

Wildtype (AB) zebrafish were obtained through natural matings from zebrafish kept in a fish facility according to Kimmel et al. (1995). Wild-type embryos were staged according to Kimmel et al (1995).

5.3.2 Immunohistocytochemistry

In situ hybridization was performed as previously described (Hammerschmidt et al, 1996; Ninkovic et al., 2005) using the following probes: *neurog1* (Korzh et al., 1998), *deltaNP63* (Kudoh et al., 2001), *her4* (Takke et al., 1999), *her5* (Müller et al., 1996) and *her3* (Bae et al., 2005). For the *her8a* probe, a 648bp fragment was cloned into pCRII-TOPO (Invitrogen)

from cDNA from 24hpf AB embryos using forward: CACTGCTTGGAAGCAAATGA; reverse: GACTTGGCGTGTGATTGATG (PCR conditions available on request). The successful clone was verified by sequencing. RNA probes were synthesized following published protocols (Hauptmann and Gerster, 1994). For immunohistochemisty the primary antibody was Anti-Hu, Mouse, (diluted 1:1500) (A21271; Molecular Probes) revealed using Cy-2.

5.3.3 RNA and morpholino injections

Capped RNAs were synthesized using Ambion mMessage mMachine Kit and embryos were injected at the one cell stage. For *her8a* overexpression, full length *her8a* was cloned into pXT7 (forward: AATAATGACGGCCTCCAACA; reverse: GGCTGCATTCATTCACCAG) and was injected at a concentration of 62.5 ng/µl. NICD overexpression was achieved by injecting capped RNA for *nic*, which encodes the NICD fragment of zebrafish Notch1 (Haddon et al., 1998; Takke et al., 1999). Morpholinos were purchased from Gene-Tools (Philomath, USA) and gripNAs were purchased from Active Motif (Carlsbad, USA). The *her8a* splice morpholino (ATGTGACATTACCTTTCGCTCCTCT) was injected at 1mM. The *her3* morpholino was injected at 0.5mM. For the combined knockdown of *her3*, *her5*, *her9* and her11 the following were used: *her3* morpholino (TGCAGCCATTGTCCTTAAATGCTCA (Bae et al., 2005); 0.75mM), *her5* gripNA (GGTTCGCTCATTTTGTGT; 0.25mM), *her9* morpholino (GTGATTTTTACCTTTCTATGCTCGC (Bae et al., 2005); 0.75mM) and *her11* grip (AGTCGGTGTGCTCTTCAT (Ninkovic et al., 2005); 0.25mM).

5.3.4 DAPT treatment

DAPT treatment was carried out according to Geling et al. (2002). Embryos were placed in 100µm DAPT (Alexis Biochemicals) and 1% DMSO dissolved in embryo medium from 50% epiboly to be fixed at the 3 somite stage or from 24hpf to be fixed at 48hpf. Control embryos received a corresponding treatment with 1% DMSO. After treatment, the embryos were fixed in 4% PFA overnight at 4°C, before being processed for in situ hybridisation.

5.4 *her* expression in the adult brain

All experiments were performed on adults of the AB/AB wild-type strain at 5-6 months. Animals were sacrificed and the brains were removed after fixation in 4% paraformaldehyde. The brains were then postfixed in 4% paraformaldehyde overnight, before being subsequently embedded in albumin-gelatine:sucrose, which had been denatured with glutaraldehyde. Cross sections of 70µm were made using a vibratome, and then the sections were washed in PBT and progressively dehydrated into methanol. In situ hybridisation was performed as published previously on embryos (110). The following mRNA in situ probes were used: *her8a* (from this study), *her3* (Bae et al., 2005), *her4* (Takke et al., 1999) and *her9* (Leve et al., 2001). Expression was revealed by staining for alkaline phosphatase activity using NBT-BCIP or Fast Red (Sigma) (Hauptmann and Gerster, 1994).

5.5 Cell labelling and counting experiments

5.5.1 BrdU labelling

BrdU was administered in accordance with Adolf et al. (2006). Briefly, we injected the fish intraperitoneally with 50 µl/g body weight with bromo-deoxy-uridine (BrdU) diluted in 110 mM NaCl. The clearance time of BrdU is approximately four hours in adult fish (Zupanc and Ott, 1999). Therefore, we injected BrdU twice, with an interval of two hours between the first and last injection. This labels cells in S phase – that is, cells undergoing DNA synthesis after mitosis at the time of BrdU exposure. Survival times ranged from 30 minutes to eight days after the last BrdU injection. Then the fish were anesthetized with Tricaine, before being killed in ice water. The brains were removed immediately, before being fixed in 4% paraformaldehyde solution at 4°C for four hours. After this, they were progressively dehydrated in MeOH, and then stored in 100% MeOH at -20°C.

5.5.2 Immunohistochemistry

Immunohistochemistry was carried out in accordance with Adolf et al. (2006). Brains were embedded in 3% agarose in PBS and sectioned cross using a vibrating microtome (HM 650 V, Microm). The sections were blocked with 0.5% Triton X-100 and 10% normal goat serum in pBS for one hour at RT. They were then incubated in primary antibodies diluted in block buffer at 4°C overnight. Primary antibodies used in this study were rabbit anti-MCM5 (1;1500, Shoojin) (Ryu et al., 2005), human anti-Hu (1:8000, Molecular probes), rat anti-BrdU (1:200, abcam) and rabbit anti-cleaved caspase (1:500, Cell Signalling). Primary antibodies were detected using species-specific secondary antibodies labelled with Cy5, Alexa 555, Alexa 488 or Alexa 647. BrdU immunostaining requires a pretreatment with 2 M HCl, followed by washes with borate buffer and PBS, before incubation in primary antibody. After the revelation, the sections were embedded in Aqua Polymount (Polyscience) and imaged using a Zeiss confocal microscope (LSM 510 META).

5.5.3 Statistics

Statistics were performed with the statistical software “R” (http://www.R-project.org), using either t-Test for samples with unequal variances or Fisher's exact test for the comparison of the proportion of 0 counts, as appropriate.

5.6 Molecular characterisation of *nad*

The methods corresponding to this section of work are described in detail in the manuscript in appendix 8.3.

6. Abbreviations

5-HT……...……....serotonin
ACh……...……..….acetylcholine
AChE……...…….acetylcholinesterase
AMPAR……...…… α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Amph……...……amphetamine
BDNF……...…….....brain-derived neurotrophic factor
bHLH……Basic-helix-loop-helix
BrdU……...……...5-bromo-2-deoxyuridine
CNS……...……Central Nervous System
CNTF.....ciliary neurotrophic factor
co-IP……...co-immunoprecipitation
COMT……...……catechol-O-methyl transferase
CPP……...……..…..conditioned place preference
CREB……...…….……..cyclic-AMP response-element-binding protein
ctnnbl .....catenin, β-like
Dcx…….…….....…….. doublecortin
dPa…….....……..dorsal pallium
dSub…….…….....…..dorsal subpallium
DAPT…….……...….N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester
DAT……...……...…...dopamine transporter
Dc.....area dorsalis telencephali pars centralis
Dd.....area dorsalis telencephali pars dorsalis
Dld.....area dorsalis telencephali pars lateralis
Dlv.....area dorsalis telencephali pars lateralis ventralis
Dmd.....area dorsalis telencephali pars medialis
Dmv.....area dorsalis telencephali pars medialis ventralis
DG.....Dentate gyrus
DMSO.....……...……Dimethyl sulfoxide
DNA…….....Deoxyribonucleic acid
DAPT……...……...N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester
EBI……European Molecular Biology Laboratory
EGF…….……epidermal growth factor
EMBL…….....European Molecular Biology Laboratory
ENU…….……...……ethylnitrosourea
EPO.....erythropoietin
FGF…….……...…….fibroblast growth factor
fgfr……...……..fibroblast growth factor receptor
FosB……...…..FBJ murine osteosarcoma viral oncogene homolog B
GABA…….……...……gamma-aminobutyric acid
GDNF……...….. glial cell line-derived neurotrophic factor
GFAP……...……glial fibrillary acidic protein
GO…….……...……..gene ontology
GST.....GST pull-down assay
Hairy/E(Spl)…….....……..Hairy and Enhancer of Split

Hd……..……..dorsal zone of the periventricular hypothalamus
Hc……central nucleus of the ventral hypothalamus
Her……...……Hairy-related
Hes……...……..Hairy and enhancer of split
Him……...……..Her5 image
Hox……...……...Homeobox protein
hpf……...……..hours post fertilisation
ICSS……..intracranial self-stimulation
IGF.....insulin growth factor
IPZ.....isthmic proliferation zone
KO…….....…….….knockout
lPa……..lateral pallium
LIF.....leukemia inhibitory factor
LTP……long-term potentiation
LTD…….long-term depression
M2H.....mammalian two-hybrid assay
MAO……....…monoamine oxidase
Mash….....mammalian achaete-scute homologue
MCM.....minichromosome maintenance deficient
MELK.....maternal embryonic leucine zipper kinase
MeOH……....……methanol
mhb……...…….....midbrain-hindbrain boundary
MPTP…….....1-mthyl-4-phenyl-1,2,3,6-tetrahydropyridine
mPa…….....medial pallium
msi.....musashi
NAc……....……nucleus accumbens
nad……*no addiction*
NCBI..... National Center for Biotechnology Information
NE…….....…….norepinephrine
NET……...……norepinephrine transporter
Ngn……...……...….. Neurogenin
NICD……...……...Notch intracellular domain
NMDAR…….. N-methyl-D-aspartate receptor
NO…….…… nitric oxide
NPCs……neural progenitor cells
OB……...olfactory bulb
Or.....orange domain
Pax……...…….Paired box transcription factor
PBS……...……..Predicted Biological Score
PCNA…….Proliferating Cell Nuclear Antigen
PCP…… phencyclidine
PCR……...….polymerase chain reaction
PDGF.....platelet-derived growth factor
PEGF.....pigment epithelium-derived factor
Per……...……..….. *period* homolog
PN……pedunculopontine nucleus
pnSub…….postcommisural nucleus of the ventral subpallium
ppm1g.....protein phosphatase 1G
pPa……...……....posterior pallium

Ppa……..preoptic nucleus
psap.....prosaposin
qPCR ……..quantitative PCR
QTG……quantitative trait gene
QTL……..quantitative trait loci
RA.....retinoic acid
RNA…….……. Ribonucleic acid
RT-PCR……..……Reverse transcription PCR
s……..……..somites
sAPP.....secreted amyloid precursor protein
SERT.....serotonin transporter
SEZ……subependymal zone
SGZ……subgranular zone
Shh.....sonic hedgehog
Sox……..…..…...……...Sry-related HMG box transcription factor
Teg.....tegmentum
TelV……..…….…..telencephalic ventricle
TGF.....transforming growth factor
TH……..……tyrosine hydroxylase
TeO…….....……...tectum opticum
TL……...torus longitudinalis
TPZ……..……..…...tectal proliferation zone
TSc.....torus semi-circularis
Val.....valvula cerebelli lateralis
Vd.....area ventralis telencephali pars dorsalis
VEGF.....vascular endothelial growth factor
Vl….....area vetralis telencephali pars lateralis
VP…….....……...ventral pallidum
vSub…….....…….ventral subpallium
VTA…….…….....…...ventral tegmental area
Vv.....area ventralis telencephali pars ventralis
wt…….....……..wildtype
y2h……..…….yeast two-hybrid assay

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8. Appendices

Appendix 8.1. Summary of yeast-2-hybrid results. Proteins in bold were chosen for further analysis.

Global PBS	Symbol	Name	GO terms - biological function; additional information	Accession no.	GID
A	***her8a***	**hairy-related 8a**	**regulation of transcription**	**NM_199624.2**	**GID: 50878286**
A	*kpna4*	karyopherin alpha 4 (importin alpha 3) (kpna4)	protein import into nucleus	NM_201305.1	GID: 41152017
A	***ppm1g***	**protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform**	**protein amino acid dephosphorylation; No functional data in zebrafish. Could be involved in the multiple cycles of phosphorylation and dephosphorylation required for splicing.**	**NM_201488.1**	**GID: 41393132**
A	***psap***	**prosaposin**	**sphingolipid metabolic process**	**NM_131883.1**	**GID: 18859264**
A	*si:ch211-219i10.1*	si:ch211-219i10.1	lipid transport; response to chemical stimulus	NM_001030062.1	GID: 71834285
A	*zgc:66331*	PREDICTED: Danio rerio zgc:66331	mitosis	XM_681303.1	GID: 68354481
B	*fam60a*	family with sequence similarity 60, member A		NM_198825.2	GID: 52546699
B	*her11*	hairy-related 11	midbrain-hindbrain boundary development; negative regulation of neurogenesis; regulation of transcription	NM_001003886.1	GID: 51468031
B	***hes6***	**hairy and enhancer of split 6 (Drosophila)**	**regulation of transcription; somitogenesis**	**NM_194400.2**	**GID: 47271428**
B	hypothetical proteinXP_6950	similar to LOC407638 protein	nucleotide and protein blast - no info; no conserved domains	XM_695066.1	GID: 68437790
B	*kpna2*	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	protein import into nucleus	NM_001002335.1	GID: 50539737
B	*mmp13*	matrix metalloproteinase	metabolic process; proteolysis	NM_201503.1	GID: 41393162

		13			
B	***her13* (previously zgc:110599)**	**hairy-related 13**	**regulation of transcription**	**NM_001017901.1**	**GID: 62955772**
B	zgc:110662	zgc:110662	protein import into nucleus	NM_001018153.1	GID: 66392218
B	***ctnnbl1* (previously zgc:77673)**	**catenin, beta like 1**	**-;** the function of this protein is yet to be determined. However, the C-terminal portion of the protein possesses apoptosis-inducing activity.	**NM_200866.1**	**GID: 41053665**
C	similar to Lepre1 protein	similar to Lepre1 protein		XM_686014.1	GID: 68440392
C	**similar to NucleoproteinTPR**	**similar to Nucleoprotein TPR**	**-; Contains TPR motifs, involved in protein-protein interactions. TPR motifs thought to be important for the functioning of chaperone, cell-cycle, transcription and protein transport complexes.**	**XM_694645.1**	**GID: 68390565**
D	*arntl1b*	aryl hydrocarbon receptor nuclear translocator-like 1b	signal transduction; photoperiodism; regulation of transcription	NM_178300.1	GID: 30231255
D	*cand1*	cullin-associated and neddylation-dissociated 1		NM_213485.1	GID: 47087304
D	*dnmt7*	DNA (cytosine-5-)-methyltransferase 7	DNA methylation	NM_001020476.1	GID: 66472505
D	*esrrd*	estrogen-related receptor delta	regulation of transcription	XM_685723.1	GID: 68392616
D	*her1*	hairy-related 1	regulation of transcription; somitogenesis	NM_131078.1	GID: 18858788
D	*her12*	hairy-related 12	Notch signalling pathway; brain development; regulation of transcription; somitogenesis	NM_205619.1	GID: 45387662
D	*her3*	hairy-related 3	regulation of transcription	NM_131080.1	GID: 18858792
D	hypothetical proteinXP_6836	PREDICTED: Danio rerio hypothetical protein LOC560265	nucleotide and protein blast - no info; no conserved domains	XM_683661.1	GID: 68390714
D	hypothetical proteinXP_6873	PREDICTED: Danio rerio hypothetical protein LOC563968	nucleotide and protein blast - no info; no conserved domains	XM_687330.1	GID: 68363075
D	*pax3*	paired box gene 3	regulation of	NM_131277.1	GID:

			transcription; xanthophore differentiation		18859206
D	*pax6a*	paired box gene 6a	regulation of transcription; hindbrain development; lens development in camera-type eye	NM_131304.1	GID: 18859208
D	*pax7*	paired box gene 7	pigmentation during development; regulation of transcription	NM_131326.1	GID: 24158479
D	*ppp2r5e1*	protein phosphatase 2, regulatory subunit B (B56)	signal transduction	NM_194412.2	GID: 41282171
D	Zebra fish - hom. of hZNF9	putative homolog of ZNF91-like Human ; [prey614210 - Zebra fish - hom. of hZNF9]		putative homolog of ZNF91-like Human ; [prey614210 - Zebra fish - hom. of hZNF9]	
D	Zebra fish - hom. of hZNF9	putative homolog of ZNF91-like Human ; [Zebra fish - hom. of hZNF9]		putative homolog of ZNF91-like Human ; [Zebra fish - hom. of hZNF9]	
D	Zebra fish - hom. of hDGKD	putative homolog of Human DGKD ; [prey614354 - Zebra fish - hom. of hDGKD]		putative homolog of Human DGKD ; [prey614354 - Zebra fish - hom. of hDGKD]	
D	Zebra fish - hom. of prey6	no match; [prey614326 - Zebra fish - hom. of prey6]		no match; [prey614326 - Zebra fish - hom. of prey6]	
D	Zebra fish - hom. of prey6	no match; [Zebra fish - hom. of prey6]		no match; [Zebra fish - hom. of prey6]	
D	*rars*	arginyl-tRNA synthetase	ATP binding	NM_200048.1	GID: 41053406
D	*sept2*	septin 2	cell cycle	BC067625.1	GID: 45709376
D	similar to 26S proteasome n	similar to 26S proteasome non-ATPase regulatory subunit 11 (26S proteasome regulatory subunit S9) (26S proteasome regulatory subunit p44.5), transcript		XM_703351.1	GID: 68437954

Appendix 8.1. Summary of yeast-2-hybrid results.

		variant 2			
D	similar to Bullous pemphigo	PREDICTED: Danio rerio similar to Bullous pemphigoid antigen 1, isoforms 6/9/10 (Trabeculin-beta) (Bullous pemphigoid antigen) (BPA) (Hemidesmosomal plaque protein)		XM_693209.1	GID: 68391675
		(Dystonia musculorum protein) (Dystonin) (LOC569802)			
D	similar to DNAtopoisomerase	PREDICTED: Danio rerio similar to DNA topoisomeraseII_beta		XM_693293.1	GID: 68391678
D	similar to Zinc finger prot	PREDICTED: Danio rerio similar to Zinc finger protein 180 (HHZ168)		XM_687093.1	GID: 68398626
D	similar to Zinc finger protein	PREDICTED: Danio rerio similar to Zinc finger protein 35 (Zinc finger protein HF.10)		XM_688351.1	GID: 68432892
D	similar to ankyrin repeat d	similar to ankyrin repeat domain 15		XM_691965.1	GID: 68361831
D	similar to carnitine palmit	carnitine palmitoyltransferase 1A		XM_684585.1	GID: 68432320
D	similar to diacylglycerol k	similar to diacylglycerol kinase, delta 130kDa isoform 1		XM_688420.1	GID: 68363095
D	similar to high-mobility gr	PREDICTED: Danio rerio similar to high-mobility group 20A		XM_688329.1	GID: 68405007
D	similar to kinesin-associat	similar to kinesin-associated protein 3		XM_684890.1	GID: 68355885
D	similar to microfilament an	Danio rerio similar to microfilament and actin filament cross-linker protein isoform a		XM_685588.1	GID: 68392944
D	similar to pentatricopeptid	PREDICTED: Danio rerio similar to pentatricopeptide repeat domain 1		XM_693706.1	GID: 68353971
D	similar to sal-like4	PREDICTED: Danio rerio similar to sal-like 4		XM_696252.1	GID: 68442392
D	similar to	PREDICTED: Danio		XM_680326.1	GID:

	slit homolog3	rerio similar to slit homolog 3			68404770
D	similar to zinc finger prot	PREDICTED: Danio rerio similar to zinc finger protein 569		XM_690612.1	GID: 68440488
D	similar toCG31756 -PA	PREDICTED: Danio rerio similar to CG31756-PA		XM_680049.1	GID: 68404750
D	similar to kaiso	similar to kaiso		XM_685882.1	GID: 68374537
D	smarcb1	SWI/SNF-related matrix associated protein		NM_131448.1	GID: 54262108
D	Zebra fish - GenMatch	unknown; [prey614119 - Zebra fish - GenMatch]		unknown; [prey614119 - Zebra fish - GenMatch]	GID: 61673598
D	Zebra fish - GenMatch	unknown; [prey614132 - Zebra fish - GenMatch]		unknown; [prey614132 - Zebra fish - GenMatch]	GID: 53748639
D	Zebra fish - GenMatch	unknown; [prey614297 - Zebra fish - GenMatch]		unknown; [prey614297 - Zebra fish - GenMatch]	GID: 54888710
D	Zebra fish - GenMatch	unknown; [prey614322 - Zebra fish - GenMatch]		unknown; [prey614322 - Zebra fish - GenMatch]	GID: 82617456
D	Zebra fish - GenMatch	unknown; [prey614424 - Zebra fish - GenMatch]		unknown; [prey614424 - Zebra fish - GenMatch]	GID: 53748639
D	Zebra fish - GenMatch	unknown; [prey614430 - Zebra fish - GenMatch]		unknown; [prey614430 - Zebra fish - GenMatch]	GID: 28412547
D	zgc:100951	zgc:100951		NM_001003622.1	GID: 57525637
D	zgc:110443	zgc:110443	regulation of Rab GTPase activity	NM_001024394.1	GID: 66773154
D	zgc:73380	zgc:73380		NM_200811.1	GID: 41387133
D	zgc:76878	zgc:76878	intracellular protein transport; protein import into nucleus, docking	NM_200905.3	GID: 47131213
D	zgc:77244	importin 7 (previously zgc:77244)		NM_207049.1	GID: 46309466
N/A	*eef1g*	eukaryotic translation elongation factor 1 gamma	translational elongation	NM_173263.1	GID: 27545276
N/A	*mta2*	metastasis associated 1 family, member 2	regulation of transcription, DNA-dependent	NM_214695.1	GID: 47550704
N/A	similar to	PREDICTED: Danio		XM_696267.1	GID:

Appendix 8.1. Summary of yeast-2-hybrid results.

	Importin alpha-1	rerio similar to Importin alpha-1 subunit (Karyopherin alpha-1 subunit) (SRP1-beta) (RAG cohort protein 2) (Nucleoprotein interactor 1)			68442406
N/A	similar to glucocorticoid m	PREDICTED: Danio rerio similar to glucocorticoid modulatory element binding protein 2		XM_692267.1	GID: 68403010
N/A	similar to leprecan1	PREDICTED: Danio rerio similar to leprecan 1		XM_682009.1	GID: 68358901
N/A	similar to transcription fa	PREDICTED: Danio rerio similar to transcription factor Her-8a		XM_688549.1	GID: 68357359
N/A	*snrp70*	U1 small nuclear ribonucleoprotein polypeptide A		NM_001003875.1	GID: 51468001
N/A	Zebra fish - GenMatch	unknown; [prey614342 - Zebra fish - GenMatch]		unknown; [prey614342 - Zebra fish - GenMatch]	GID: 61673602
N/A	zgc:112226	zgc:112226	proteolysis	NM_001024409.1	GID: 66912206

Appendix 8.2 Summary of embryo manipulation results

Material injected	Stained for/stage examined	Phenotype (compared to the appropriately treated control)	Proportion showing phenotype (percentage)	Proportion delayed (did not reach stage of analysis) (percentage)	n
Morpholino-mediated knockdown					
her8a MO el (1mM)	*neurog1* at 3s	Ectopic *neurog1* expression at rhombomeres 2 and 4	35/52 (67.3%)	7/52 (13.5%)	52
her8a MO e1 (1mM)	*her8a* at 3s	Complete loss of *her8a* expression	30/42 (71.4%)	5/42 (11.9%)	42
her8a MO e1 (1mM)	*her5* at 3s	No phenotype	-	4/38 (10.5%)	48
her8a MO e1 (1mM)	*her3* at 3s	No phenotype	-	2/11 (18.2%)	37
her3 MO (0.5mM)	*neurog1* at 3s	Ectopic *neurog1* expression at rhombomeres 2 and 4 (previously published)	28/53 (52.8%)	3/53 (5.7%)	53
her3 MO (0.5mM)	*her8a at 3s*	No phenotype	-	2/24 (8.3%)	24
her3 MO (0.5mM) and *her8a* MO el (1mM)	*neurog1 at3s*	Ectopic *neurog1* expression at rhombomeres 2 and 4 – no additional phenotype	16/26 (61.5%)	6/26 (23.1%)	26
her3, *her5*, *her9* and *her11* knockdown (see methods)	*her8a* at 3s	No phenotype	-	4/28 (14.3%)	28

her3, *her5*, *her9* and *her11* knockdown (see methods)	*neurog1* at 3s	Ectopic *neurog1* in progenitor zones at and surrounding mhb (C. Stigloher, unpublished)	14/27 (51.9 %)	8/27 (29.6%)	27
her8a MO e1 (1mM)	*sox19a*	No phenotype	-	2/9 (22.2%)	9
her8a MO e1 (1mM)	*sox19b*	No phenotype	-	4/9 (44.4%)	9
her8a MO e1 (1mM)	*sox21*	No phenotype	-	2/11 (18.2%)	11
her8a MO e1 (1mM)	*sox2*	No phenotype	-	1/9 (11.1%)	9
her8a MO e1 (1mM)	*sox3*	No phenotype	-	2/9 (22.2%)	9
sox2/3 (1mM), sox19a (1mM), sox19b (0.25mM), sox21b (1mM) MOs	*neurog1 at 3s*	Ectopic *neurog1* in mesoderm (individually no phenotype)	10/25 (40%)	4/25 (16%)	25
sox19a, sox19b, sox21b, sox2/3 MOs	*her8a at 3s*	Decrease in *her8a* expression	18/20 (90%)	2/20 (10%)	20
Overexpression experiments: capped RNA injection					
her8a-pXT7 (62.5ng/µl)	*neurog1* at 3s	Complete loss of *neurog1* expression	17/30 (56.7%)	8/30 (26.7%)	30
her8a-pXT7 (62.5ng/µl)	*her8a* at 3s	No phenotype	-	5/29 (17.2%)	29
her8a-pXT7 (62.5ng/µl)	*her3* at 3s	No phenotype	-	4/29 (13.8%)	29
her3 (60ng/µl)	*neurog1* at 3s	Hans et al. (2004) reported a complete loss of *neurog1* expression. I was unable to recreate this.	-	1/21 (4.8%)	21

her3 (100ng/µl)	*neurog1* at 3s	see above	-	5/26 (19.2%)	26
her3 (180ng/µl)	*neurog1* at 3s	see above	-	18/18 (100%)	18
NICD	*her8a* at 3s	Overexpression throughout the embryo	17/19 (89.5%)	2/19 (10.5%)	19
NICD	*neurog1* at 3s (with *tp63* to delimit neural plate)	Loss of expression throughout the embryo (previously published…)	14/18 (77.8%)	2/18 (11.1%)	18
NICD	*her4* at 3s	Overexpression throughout the embryo	12/13 (92.3%)	1/13 (7.7%)	13
Engd-*her8a* (150ng/µl)	*neurog1* at 3s	No phenotype	-	3/15 (20%)	15
her8a-delta basic (150ng/µl)	*neurog1* at 3s	No phenotype	-	8/24 (33.3%)	24
her8a-VP11x2 (150ng/µl)	*neurog1* at 3s	No phenotype	-	9/33 (27.3%)	33
Treatments					
DAPT (50% epiboly to 3 somites)	*neurog1* at 3s	Increase in *neurog1* positive neurons in the proneural clusters (previously reported in Geling et al., 2002)	15/21 (71.4%)	2/21 (9.5%)	21
DAPT (50% epiboly to 3 somites)	*her8a* at 3s	No phenotype	-	3/18 (16.7%)	18
DAPT (24hpf to 48hpf)	*her8a* at 48hpf	Complete loss of *her8a* expression throughout the embryo	25/26 (96.2%)	1/26 (3.8%) (approximately 24hpf stage – still had *her8a* staining)	26
DAPT (24hpf to 48hpf)	*her4* at 48hpf	Ectopic expression in eye field	18/18 (100%)	-	18

		of expression in spinal chord; no change in the telencephalon			
DAPT (24hpf to 48hpf)	*her9* at 48hpf	Decreased expression. Some retained in the midline.	16/16 (100%)	-	16
DAPT (24hpf to 48hpf)	*her15* at 48hpf	Ectopic expression in the eye.	15/15 (100%)	-	15

Appendix 8.3: Article currently under revision at Genome Biology.

Zebrafish reward mutants reveal novel transcripts mediating the behavioral effects of amphetamine

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Appendix 8.3. Zebrafish reward mutants reveal novel transcripts mediating the behavioural effects of amphetamine

Abstract

Addiction is a pathological dysregulation of brain reward systems, determined by several complex genetic pathways. The conditioned place preference (CPP) test provides an evaluation of the effects of drugs in animal models, allowing the investigation of substances at a biologically relevant level with respect to reward. Our lab has previously reported the development of a reliable CPP paradigm for zebrafish. Here, this CPP test was used to isolate a dominant ENU-induced mutant, *no addiction* (*nad*^{dne3256}), which fails to respond to amphetamine, and which we used as an entry point towards identifying the behaviorally- relevant transcriptional response to amphetamine. Through the combination of microarray experiments comparing the adult brain transcriptome of mutant and wild-type siblings under normal conditions, as well as their response to amphetamine, we identified genes that correlate with the mutants' altered CPP behavior. In addition to pathways classically involved in reward, this gene set shows a striking enrichment in transcription factor-encoding genes classically involved in brain development, which later appear to be re-used within the adult brain. We selected a subset of them for validation by quantitative PCR and in situ hybridization, revealing that specific brain areas responding to the drug through these transcription factors include domains of ongoing adult neurogenesis. Finally, network construction revealed functional connections between several of these genes. Together, our results identify a new network of coordinated gene regulation that influences or accompanies amphetamine-triggered CPP behavior and that may underlie the susceptibility to addiction.

Appendix 8.3. Zebrafish reward mutants reveal novel transcripts mediating the behavioural effects of amphetamine

Background

Addiction, which can be broadly defined as a pathological state characterized by the compulsive seeking and usage of a drug in spite of adverse consequences, is a major societal problem. In the USA alone, more than 23 million Americans are concerned, with societal costs reaching 1.4 million dollars over the life of each addict (from: The Economic Cost of alcohol and drug abuse in the United States 1992-1998; NIDA, 2001). Addictive drugs include a large number of substances (such as stimulants, alcohol and opiates), acting through different cellular mechanisms, but which all trigger a sequence of widespread long-lasting consequences on brain physiology, most of which are only partially understood. The complexity of these plastic events makes it difficult to efficiently care for patients, and current treatments have little power to avoid relapse. As a consequence, a major goal of drug abuse research is to identify the key molecular mechanisms underlying the development of compulsive drug use which may then be medically targeted for better treatments.

The mechanisms underlying drug addiction utilize a succession of physiological responses that begin with activation of the brain's reward pathway, common to all drugs of abuse. The reward system, largely based on dopamine signaling projecting to forebrain centers (Wise, 2002), signals a pleasurable experience, which then tends to be repeated. The transition from drug use to addiction (Vanderschuren and Everitt, 2004) occurs gradually and involves both neuro- and synaptic plasticity. These long-lasting adaptive changes persist even after withdrawal of the drug, and they are likely to underlie the persistent tendency to relapse (Bossert et al., 2005). In addition, several other circuits - in particular the stress axis and the learning and memory circuitry - have been implicated in the reinforcement of drug use or addiction and in the cognitive processes underlying addiction (Kelley, 2004). One powerful approach to understand which molecular

alterations contribute to the development and expression of the successive addiction-related behaviors has been the use of microarray expression profiling. Combined with the in silico assembly of regulatory networks, this high-throughput analysis can provide a comprehensive picture of the changes in gene expression that may underlie the different steps towards drug addiction. In the case of psychostimulant drugs for example, microarray analyses have demonstrated the occurrence of important transcriptional changes that differ over time, clearly distinguishing acute from chronic drug use or withdrawal. In models as varied as human post-mortem brains from cocaine abusers and mice or rats of different genetic backgrounds, these changes were related to molecular pathways controlling neurotransmitter signaling (including a downregulation of the dopamine D2 receptor), signal transduction, ion-gated channel activity, cytoskeletal structures, extracellular matrix remodeling, synaptogenesis, axonal dynamics and cell metabolism ((Mash et al., 2007b), (Zhang et al., 2005b), (Yamamoto et al., 2004), reviewed in (Rhodes and Crabbe, 2005), (Yuferov et al., 2005)).

Because a major step in the development of addiction is the switch from drug use to drug abuse, we aimed to gain insight into the mechanisms triggering the initiation of addictive behavior. To this aim, we focused on commonalities in the effect of abused drugs, hence on their early effect on the reward pathway. Based on previous observations demonstrating that the response of the reward system increases with expectancy (thus it is subject to auto-amplification) (Schultz, 2002), we reasoned that a major susceptibility factor in the transition from drug use to abuse might be the intensity of the initial reward response. In order to narrow-down transcriptional approaches to this process, recent analyses compared the transcriptional effects of several drugs (Lehrmann et al., 2006b), or made use of mice carrying alterations in the function of genes postulated to be relevant to reward. For example, the transcriptional effects of cocaine were compared in mice lacking the DA D1 receptor (necessary for the sensitization to cocaine) versus their wild-type

siblings (Zhang et al., 2005b), in mice overexpressing the immediate early transcription factors CREB or ΔfosB (both of which are involved in mediating the acute effects of cocaine) (Winstanley et al., 2007) or in mice knocked-out in Cdk5 (a downstream target of ΔfosB) (Bibb et al., 2001). We aimed to provide unbiased insight into this question, without a priori selection of a regulatory pathway, while remaining clearly associated with the reward behavioral output. To achieve these goals, we initiated a functional study of the reward pathway in zebrafish, a vertebrate model amenable to random mutagenesis and behavioral screening. Reward behavior is an ancestral behavior, conserved throughout vertebrate phyla, and the underlying neurotransmitter pathways are shared between species ((Bretaud et al., 2007; Lett and Grant, 1989; Levens and Akins, 2001; Ninkovic et al., 2006a)). We chose to use the psychostimulant amphetamine, as it directly stimulates the reward pathway (largely via altering the function of the dopamine transporter Dat (Sulzer and Edwards, 2005) that elicits limited physical dependency, and on the behavioral assay known as Conditioned Place Preference (CPP)). This test, in which association with the pleasurable effect of a drug modifies an animal's choice for a specific environmental cue, is classically taken as a read-out of the functionality of the reward system (Tzschentke, 2007). Using amphetamine, we recently developed a robust assay for drug-induced CPP behavior in adult zebrafish, and demonstrated the role of ACh signaling in the sensitivity to amphetamine-induced reward ((Ninkovic and Bally-Cuif, 2006; Ninkovic et al., 2006a)). Here, relying on evidence suggesting genetic components in the susceptibility to addiction (reviewed in (Crabbe, 2002; Everitt et al., 2008; Volkow and Li, 2004)), we used this assay in an ENU mutagenesis screen, successfully isolating an amphetamine-resistant mutant in the CPP test, *no addiction* (*nad*^{dne3256}, thereafter referred to as *nad*). This mutation is dominant and *nad* heterozygotes fail to change their place preference upon repeated amphetamine administration. In zebrafish, amphetamine does not trigger a locomotor response (Ninkovic et al., 2006a), and lack of CPP is the only phenotype that we could associate with the *nad* mutation to date. We next used this

mutant in a three-step expression profiling paradigm comparing the transcriptional response of wild-type animals upon CPP-stimulating amphetamine administration with that of *nad* mutants receiving either drug or a saline control solution. We discovered a set of 139 genes that respond to amphetamine in wild-type animals, but respond differently in *nad* mutants without being altered under basal conditions in either genotype. In addition to genes involved in pathways classically associated with reward, this gene set shows a striking enrichment in transcription factors, which are specifically known for their involvement in brain development. We validated a subset of these genes using qPCR and in situ hybridization, thereby revealing an association of these gene expressions with neurogenic zones of the adult brain, which is also apparent in the mouse. Using an in-house developed database linking zebrafish genes to information on orthologous gene interactions, we could further demonstrate that most of these genes contribute to a common regulatory network. Together, our results identify a pattern of coordinate gene regulation that may underlie or accompany the development of CPP behavior upon amphetamine administration and hence may contribute to generating a susceptibility background towards the development of addiction.

Results

The mutant *nad*^{dne3265} fails to respond to amphetamine-induced reward

To recover mutants of the amphetamine response, we designed an ENU mutagenesis screen making use of the amphetamine-based conditioned place preference (CPP) test for adult zebrafish (Ninkovic and Bally-Cuif, 2006). Briefly, in this test, the psychostimulant amphetamine is provided in association with the initially non-preferred side of a two-color tank. Following repeated administration, amphetamine causes a switch in the place preference of the fish: even in

the absence of drug, the animal will now prefer the amphetamine-paired side of the tank. As previously demonstrated using adults heterozygous for the *ache*^{sb55} mutation, this test is robust enough to detect dominant mutations affecting amphetamine-triggered preference (Ninkovic et al., 2006a). To recover new dominant mutations of this type, we screened F1 animals generated from ENU-treated F0 males for their place preference response to amphetamine. Potential mutants were then out-crossed to wild-type fish and their F2 progeny was retested at adulthood. From 396 F1 animals tested (corresponding to 396 genomes), 4 animals failed to change their place preference upon amphetamine administration while showing normal initial place preference without drug (not shown). One of these potential mutants transmitted this phenotype to 50% of its progeny, following the expected Mendelian distribution for dominant genetic traits (Fig.1A). To date, this transmission has been stable over more than 5 generations and is detectable equally well in both the AB and the polymorphic AB/Tü background (Ninkovic and Bally-Cuif, 2006), arguing for a bona fide dominant mutation. Importantly, the initial place preference in mutants does not differ from that of their siblings (Fig.1B), demonstrating their normal response to the visual cues of the test tank. Following drug treatment, amphetamine brain content is also similar in mutant fish and their siblings (not shown). We name this mutation *no addiction* (*nad*^{dne3256}, thereafter referred to as *nad*).

A distinct gene expression signature underlies the abnormal behavioral response of *nad* mutants to amphetamine

Previous experiments based on candidate gene or microarray analysis demonstrate that amphetamine treatment has an impact on gene expression (for a review see (Yuferov et al., 2005)). These gene expression changes are likely to mediate or reflect a large part of amphetamines' actions on multiple biological processes, one of which is to activate the reward pathway. The design of our mutant screen further implies that the effect of amphetamine on CPP

development is impaired in *nad* (see Discussion for the possible behavioral meanings of *nad*). Thus, the changed transcriptional response of *nad* to the drug can help identify the genes meaningful to the response to amphetamine.

We designed three microarray comparisons to specifically isolate such genes (Fig.2A) (see GEO database for the complete genes lists). In a first comparison, we found 1214 genes to be differentially expressed between wild-type fish that received amphetamine treatment triggering CPP versus fish that received a control, saline treatment (experimental conditions identical to those described above) (microarray experiment 1, "wt+/wt-") (Fig.2A, purple group). To extract genes meaningful to CPP development from this pool, we next identified the transcripts that were differentially affected by amphetamine in *nad* versus their wild-type siblings. Analysis of the microarray data for this second comparison showed that 958 genes were differentially expressed between mutants and wild-type siblings upon amphetamine treatment (experiment 2, "mut+/sib+") (Fig.2A, pink group). However because these might also include basal transcriptional differences between mutants and wild-type fish (i.e. transcriptional differences that are not triggered by amphetamine administration), not related to the abnormal behaviour of the mutants, we performed a third microarray comparison between mutants and their siblings without amphetamine (experiment 3, "mut-/sib-") (Fig.2A, green group). We found 1224 genes to be differentially expressed under these conditions, which were then taken to represent the basal differences between the mutants and their siblings. Of these, 356 were also differentially expressed in the experiment "mut+/sib+" and were then subtracted from this group to recover genes characterizing the different response of mutants versus siblings to amphetamine. This subtraction resulted in the pool "mut+/sib+ minus mut-/sib–". The intersection of the pools "mut+/sib+ minus mut-/sib–" and "wt+/wt-" was taken to form the "reward pool" - that is genes that both characterize the wild-type response to amphetamine and that display altered response

(i.e. they respond less or more than in wt) in the mutant, correlating with the failure of CPP in this genotype. This pool comprises 139 genes, which are listed and functionally annotated in Suppl. Table 1.

Of the 139 genes in the reward pool, 17% were upregulated in both "mut+/sib+" and "wt+/wt-" (Fig.2B). Transcription of these genes is increased in wild-type fish upon amphetamine treatment and excessively increased in the mutants. Conversely, 24% of the 139 genes were down-regulated in both experiments; hence their transcription is normally down-regulated upon amphetamine, and is excessively down-regulated in the mutants. Finally, a majority of the genes (59% of 139) responded to amphetamine in an opposite manner between wild-type and mutant fish (24% of the 139 genes were up-regulated in mut+ compared to sib+, but downregulated in wt+ compared to wt-, and 35% were down-regulated in mut+ compared to sib+ and up-regulated in wt+ compared to wt-). These genes fail to be down- or up-regulated, respectively, in the mutants upon amphetamine treatment.

The reward pool is significantly enriched in transcription factor-encoding genes

In order to further investigate the mechanisms involved in reward, gene ontology (GO) enrichment analyses categorizing the genes in the organizing principle "biological process" were performed on each of the individual experiments and the reward pool (Fig.3A). We found that the reward pool contains a high proportion of genes encoding functions previously related to reward or the transition to addiction such as neurotransmitter signaling pathways, ion channels and regulators of neuronal and synaptic plasticity (see Suppl. Table 1 and Discussion). In order to characterize processes specific to the rewarding effects of amphetamine, we also searched for particular enrichments in the reward pool over the other three gene sets. The most striking result was that the term "transcription" was enriched across all groups, and it displayed a further relative

increase for the reward pool. This was also the case for the term "development", and in fact, both superordinate categories largely overlapped in their gene content (see Fig.3B). Thus the involvement of transcription factors previously recognized for their relevance in developmental processes distinguishes the reward response to amphetamine (and its failure in *nad*) over other transcriptional effects of amphetamine treatment.

Amphetamine-responding genes can be validated by quantitative PCR and classified as acute and/or chronic responders

The individual genes annotated in the zebrafish genome (release zv7, www.ensembl.org and www.zfin.org) and corresponding to each term for the reward pool are listed in Fig.3B (see Suppl. Table 1 for their respective fold-change in the different array experiments). We chose 10 genes for validation based on gene ontology enrichment analysis and literature searches: *ahr1a*, *dlx1a*, *emx1*, *foxg1*, *gfi1b*, *her15*, *lhx8*, *slc6a5*, *sox9a* and *tbr1* (Fig.3B). Because developmental transcription factors had not been recognized as a signature of the behavioral response to amphetamine in previous studies, our selection was largely biased towards this category: nine of the chosen genes encode transcription factors (*ahr1a*, *dlx1a*, *emx1*, *foxg1*, *gfi1b*, *her15*, *lhx8* (previously *lhx7*), *sox9a* and *tbr1*), four of which have been assigned the GO term "development" (*her15*, *foxg1*, *emx1* and *dlx1a*). In addition to their generally prominent role during brain development, strong arguments to choose these genes were (i) the maintenance of expression of their orthologues in the adult mammalian brain (respectively in mouse *Ahr*, *Dlx1*, *Emx1*, *Foxg1*, *Gfi1*, *Hes5*, *Lhx8*, *Sox9* and *Tbr1*) (Cobos et al., 2005; Hevner et al., 2006; Hong et al., 2007; Huang et al., 2003; Ohtsuka et al., 2005; Shen et al., 2006; Tsuda et al., 2005; Zhao et al., 2003) (and the Allen brain atlas) suggesting an extended role in controlling brain physiology, and (ii) their comparable expression patterns in both mouse (Englund et al., 2005; Gorski et al., 2002; Marin et al., 2000; Mori et al., 2004; Moroy, 2005; Pompolo and Harley, 2001; Shinozaki et al.,

2002; Zhao et al., 2003) and zebrafish ((Chiang et al., 2001; Ellies et al., 1997; Kawahara and Dawid, 2002; Miyake et al., 2005; Mueller et al., 2008; Stigloher et al., 2006) and our unpublished data), at least during brain development, arguing for conserved functions in these species. In addition, we chose to test *slc6a5* as a representative of the neurotransmitter pathway genes recovered in the reward pool. *slc6a5* encodes the glycine neurotransmitter transporter GlyT2, which is involved in the reuptake of glycine at the synapse.

In a first step, expression of these genes in the wild-type adult brain was confirmed using in situ hybridization. All 10 transcripts gave strong signals in the brain, including the telencephalon (Suppl. Fig.1). Specifically, the expression of *gfi1b* and *her15* is restricted to the ventricular areas of the telencephalon (Suppl. Fig.1E,F), diencephalon, and midbrain (not shown). *dlx1a*, *emx1*, *foxg1*, *lhx8*, *slc6a5*, *sox9a* and *tbr1* are expressed in restricted areas of the brain including subdomains of the pallium and/or subpallium in the telencephalon (Suppl. Fig.1B-D,G-J). Overall, the regional expression of these genes is in keeping with their known expression in the adult mammalian brain (see Discussion). *ahr1a* is expressed ubiquitously throughout the brain (Suppl. Fig.1A and not shown).

Next, quantitative PCR was used to validate the differential expression of seven of these ten genes upon amphetamine administration (*emx1, foxg1*, *gfi1b*, *her15*, *lhx8*, *slc6a5* and *sox9a*). Five of these genes (*emx1, foxg1*, *her15*, *slc6a5* and *sox9a*) were first re-tested on the original RNA used for the microarrays. All were differentially regulated in the same direction as in the microarray for both wt+/wt- and mut+/sib+, thus validating our microarray experiments (Fig. 4A, B) (see also Suppl. Table 2). We next tested all genes in new RNA samples. Our experimental design for the arrays involved 4 injections of amphetamine alternating with 3 doses of saline solution, with the last amphetamine injection given 30 minutes before death. We hypothesized that this would allow us to identify genes reacting to any or just acute or chronic amphetamine administration. As previous studies showed differences in the reaction of transcriptional levels to

these different treatments (Sokolov et al., 2003b), we conducted qPCR experiments on the 7 above-selected genes using brains from fish that had been injected once (acute) or 18 times (chronic) with amphetamine, once daily, with the last administration 30 minutes before death. The results are depicted in Fig.4C,D (see also Suppl. Table 2). *emx1* and *gfi1b* appeared differentially expressed upon acute amphetamine administration, while there was no difference in *foxg1, her15*, *lhx8*, *sox9a* and *slc6a5* expression between acute-treated and untreated samples. However, all seven genes were differentially expressed upon chronic amphetamine administration, always in the same direction as in the arrays. These results further validate our arrays and, in addition, suggest that the amphetamine administration procedure used to trigger a CPP response in this work is closer to a chronic than to an acute paradigm.

A subset of the reward pool genes is visibly modulated in situ by amphetamine

As demonstrated above, qPCR using total RNA extracted from whole brains was used to validate and extend the results of our microarrays. However, this approach does not provide information as to which regions of the brain are transcriptionally affected by the drug. In situ hybridization was next performed on brain sections of fish chronically injected with amphetamine or saline solution (once a day, for 18 days). Of the 10 genes selected above, the expression patterns of *foxg1*, *gfi1b*, *her15* and *lhx8* were visibly changed upon amphetamine administration. The expression of *gfi1b* and *her15*, which characterize the ventricular zone in all brain subdivisions, was completely and consistently down-regulated, throughout the brain (Fig. 5A-D and Suppl. Fig.2A-D). The expression of *foxg1* was reduced in the ventrolateral thalamic nucleus, and eliminated at the midline in the ventral zone of the periventricular hypothalamus and the parvocellular preoptic nucleus – no expression changes were detected throughout the remainder of the brain (Fig.5E-F and Suppl. Fig.2E-F). The expression of *lhx8* was also much reduced in this latter domain upon amphetamine treatment, but was unchanged elsewhere in the brain (Fig.

5G-H and Suppl. Fig. 2G-H). The reduction or increase of expression of the other six selected genes, indicated by the array and the qPCR, was not visible using in situ hybridization (not shown). This may occur because in situ hybridization is not a quantitative technique, and so large changes in expression are required before they can be observed. Together, these results highlight that ventricular domains of the adult brain are major areas responding to an amphetamine administration paradigm activating the reward pathway, and identify *gfi1b*, *her15*, *lhx8* and *foxg1* as prominent transcriptional targets in these domains.

We finally aimed to determine whether genes of the reward pool could be functionally connected. We developed a database (ZFISHDB) linking zebrafish genes to functional annotations and relationships via the STRING database. From the 139 genes of the reward pool, 53 could be attributed to cluster of orthologous genes. 25 interactions were found between 18 of these genes (Fig.6). In particular, 8 of the transcription factors, of which 5 have demonstrated roles in brain development (Dlx1a, Emx1, Lhx8, Sox9a and Tbr1), could be functionally connected, suggesting that amphetamine may re-use a developmental network in mediating reward in the brain.

Discussion

A major question in the field of drug addiction remains to characterize the transcriptional changes underlying the switch from drug use to drug abuse. In this study we used an unbiased paradigm to identify a subset of genes involved in reward activation and its behavioral output. Our approach does not rely on the prior selection of a particular pathway, but rather on a mutant whose only phenotype is the lack of CPP behavioral response to amphetamine. Although we have not yet identified the mutation underlying this phenotype, the *nad* mutant allowed us to extract a subset

of 139 genes from the general transcriptional response to amphetamine that respond abnormally to amphetamine in the mutants, correlating with the failure to develop CPP. The transcriptional regulation of these genes is therefore associated with reward-triggered CPP behavior. We validated the microarray using both qPCR and in situ hybridization, thereby identifying neurogenic areas as potentially significant for the response to amphetamine. In contrast to previous studies, our analysis highlighted for the first time the predominance of transcription factors in the response to amphetamine. These genes have been recognized for their function during brain development in both zebrafish and mouse, and are also expressed in the adult brain, pointing to the re-use of a developmental network as a potentially important component of reward behavior.

Behavioral significance of the reward pool

Based on a subset of genes recovered in our array, we used qPCR to show that our experimental conditions mimic chronic amphetamine administration. These genes therefore represent early but not acute transcriptional changes induced by the drug. Our experimental design also allowed us to focus on a biologically relevant dose of amphetamine with regard to activation of the reward pathway. Finally, the non-response of *nad* mutants suggests that the expression changes recovered are, in part, linked to CPP behavior. Several parameters underlie this behavior and might be altered in *nad*, such as the functionality of the reward pathway itself and the associative learning process involved in CPP, but also changes in tolerance or sensitization to rewarding or motivational events. We have not noticed any other behavioral or morphological alterations in *nad*, and also failed to observe differences in gross neuroanatomy and the organization of several neurotransmitter systems in this mutant (including dopamine and serotonin, revealed by TH and 5HT immunocytochemistry) (not shown). Nevertheless, *nad* animals may exhibit yet other deficient responses to amphetamine which might become apparent could we test later stages of

the addiction process, such as the maintenance of drug use, withdrawal or relapse. Whether *nad* mutants are also resistant to other addictive drugs primarily acting through different molecular cascades than psychostimulants, such as opiates (Lau et al., 2006), and whether the genes of the reward pool are correlatively also transcriptionally modified upon administration of these drugs, remain further very important questions. It will be essential to assess these points in the future to better connect the genes of the reward pool to behavioral function.

Identification of long-lasting amphetamine-induced transcriptional changes with no indication of toxic effects

Importantly, we did not find any evidence of genes linked to cell-stress or cell-death, either in individual experiments or in the reward pool. This is in accordance with other microarray expression profiling publications, such as (Sokolov et al., 2003b), which found little evidence of such genes upon chronic drug treatment, while many were recovered upon acute administration of psychostimulants (and other drugs like morphine (Korostynski et al., 2007)), and which may be due to the direct neurotoxic effects of amphetamine or cocaine. Likewise, immediate early transcription factors such as Erg2, Krox24, c-fos, c-jun and CREB, which are often transiently up-regulated following administration of drugs of abuse (reviewed in (Rhodes and Crabbe, 2005)), were neither enriched in individual experiments nor in the reward pool. This confirms that our gene sets reflect long-lasting rather than acute transcriptional changes resulting from amphetamine action and may mediate the link to the different aspects of addiction. The category of genes related to the biological function "response to stimulus" was enriched upon drug administration over saline in both wild-type and mutants. However these genes were filtered out in the reward pool, confirming that they reflect a pharmacological response to the administration of chemical compounds that is unlikely to be altered in *nad* and so might not be involved per se in the development of behavioral alterations upon drug taking.

We decided to extract RNA from whole brains, rather than choosing specific anatomical regions. This approach was based on several considerations. Firstly, in addition to acting on the dopaminergic and serotonergic systems, amphetamine raises extracellular levels of glutamate (Del Arco et al., 1999) and noradrenaline (Florin et al., 1994) and these circuits are widely distributed throughout the brain. Secondly, the use of zebrafish makes it difficult to precisely predict where relevant expression changes are to be expected. Although the neurochemical aspects of reward behavior, including CPP, are evolutionarily conserved (Bretaud et al., 2007; Gerlai et al., 2000; Kily et al., 2008; Lau et al., 2006; Ninkovic et al., 2006b), some of the main neurotransmitter pathways involved in these behaviors show divergent spatial organization between species. For example, the dopaminergic neurons projecting to the zebrafish subpallium (hypothesized to be an equivalent of the mammalian basal ganglia including the NAc) are located in the diencephalic posterior tuberculum, unlike in mammals where these neurons lie in the ventral tegmental area of the midbrain (Rink and Wullimann, 2001). Likewise, the zebrafish brain, as in many other vertebrate classes (Challet et al., 1996; Cozzi et al., 1991; Rodrigues et al., 2008), harbors widespread serotonergic clusters as apposed to the single mammalian raphe nucleus (Kaslin and Panula, 2001). However, as discussed below, we complemented our microarray experiments with in situ hybridization in order to allow us to investigate spatial changes in the expression of recovered transcripts and to identify relevant brain areas responding to amphetamine.

Transcriptionally regulated pathways and reward behavior

Most microarray analyses of reward and addiction to psychostimulants have been conducted in rodents. In addition, one transcriptome analysis of the adult zebrafish brain was recently published, comparing the effects of ethanol and nicotine during withdrawal (Kily et al., 2008). It

is not possible to analyze all these results side by side given the variety of drugs and drug administration protocols used and the length of time allowed following drug exposure. Nevertheless, a general outcome was the response to chronic drug use of molecular pathways controlling neurotransmitter signaling (including receptors, transporters and signal transduction components), ion channels and regulators of neuronal activity and plasticity events such as synaptic function or extracellular matrix remodeling (Lehrmann et al., 2006a; Mash et al., 2007a; Rhodes and Crabbe, 2005; Sokolov et al., 2003a; Winstanley et al., 2007; Zhang et al., 2005a). Our manual annotation of the 139 reward pool genes allowed us to identify related mammalian genes in most cases (84/139), and to postulate a function based on gene homology or on predicted protein structure for an additional 8 genes (92/139), so that our data can be directly compared to previous work. Of the functionally annotated genes of the reward pool, 28 belong to the categories above and 14 have been already linked to reward or addiction (see Suppl. Table 1).

Affected genes related to neurotransmission include the epsilon subunit of the nicotinic ACh receptor (*chnre*), glycine transporter 2 (*slc6a5,* formerly *glyT2*), and *LOC793458*, encoding peptide YYb (PYYb) (Sundstrom et al., 2008). All three pathways have been directly or indirectly implicated in reward (Kerns et al., 2005). We found *chnre* expression to be increased upon amphetamine administration in wild-type and excessively increased in *nad* animals. Therefore, amphetamine may confer enhanced excitability properties to ACh target neurons via a novel composition of the AchR, which could be linked to the development of the CPP response. Glycine is a major modulator of NMDA receptor-mediated signaling and glutamate neurotransmission is a determining factor in psychostimulant (and other) addictions (for reviews: (Feltenstein and See, 2008; Gass and Olive, 2008; Kalivas, 2007)). It has also been implicated in the regulation of neuronal differentiation, neural network plasticity and synapse dynamics. We found that *slc6a5/glyT2* is increased in wild-type and excessively increased in *nad* animals upon

amphetamine administration. Hence chronic amphetamine administration may modify the amount of glycine at the synapse via Slc6a5 activity, with possible consequences on the development or reinforcement of amphetamine-triggered reward. In mammals, PYY antagonizes the orexigenic and anxiolytic effect of Neuropeptide Y, which can itself elicit CPP reward behavior (Brown et al., 2000). We observed *pyy-b* expression to be down-regulated by amphetamine in wild-type but not *nad* animals. Down-regulation of *pyy-b* could reinforce the activity of NPY, thereby contributing to the development of reward, while its lack of response in *nad* might mediate the resistance of this mutant to CPP behavior.

Seven genes encoding proteins related to axonal or synaptic dynamics were also recovered in the reward pool (Suppl. Table 1). Among these, two belong to families that may be directly relevant to addiction or drug use: *Dr. 83111*, encoding a protein highly similar to Neuregulin 1 (NRG1) and a Drebrin-like protein-encoding gene (*Dr. 76820*). NRG1 signaling plays a prominent role in synapse plasticity in the mature brain by controlling excitatory and inhibitory synaptic transmission (Britsch, 2007; Mei and Xiong, 2008; Zhong et al., 2008), which might underlie the propensity towards drug abuse (Coyle, 2006). In humans, it has also been identified as a susceptibility factor for schizophrenia, a disease often co-morbid with substance use disorder. We found that NRG1 was strongly up-regulated by amphetamine in wild-type animals, and massively down-regulated in *nad*. This differential response may play a role in the different CPP behavioral response of *nad*. Drebrin, an F-actin-binding protein enriched in dendritic spines, is essential for spine morphogenesis and activity-dependent synaptic targeting of NMDA receptors (Lippman and Dunaevsky, 2005; Sekino et al., 2007; Takahashi et al., 2006; Takahashi et al., 2003). Both dendritic spine morphology and density in the VTA, NAc and motor cortex are altered by amphetamine and cocaine (Robinson and Kolb, 1999). We found that expression of Drebrin is increased upon amphetamine administration in wild-type animals, but fails to be upregulated

under the same conditions in *nad* mutants, suggesting that altered changes in dendritic spine remodeling accompany the resistance of *nad* to amphetamine.

Genes encoding components of the dopamine (DA) pathway were not identified in our experiment, although amphetamine is considered to primarily increase extracellular DA levels in the forebrain (Riddle et al., 2005; Williams and Galli, 2006). In drug-addicted subjects, the concentration of DA receptors (namely D2) is lowered on the cell surface as revealed by imaging studies (Volkow et al., 2007). However, corresponding changes in gene expression have not been consistently reported, suggesting that the modulation of the DA pathway may not occur at the level of transcription (Rhodes and Crabbe, 2005). Alternatively, microarray sensitivity may be insufficient to detect functionally relevant but small amplitude changes in the expression of weakly expressed factors such as DA signaling components (Yuferov et al., 2005). In support of this, although our microarray chips contained a large representation of genes encoding transporters, receptors, synthesis and metabolism enzymes for most neurotransmitters (including dopamine, glutamate, noradrenaline, 5HT, NPY, acetylcholine, glycine and opiates), we only obtained reproducible hybridization indicating sufficient expression for a few of these genes (see GEO Database). From these, apart from *chnre* and *slc6a5* (discussed above), only the *glutamate transporter 5A* and *neuropeptide Y receptor Y7* showed a significantly modified expression upon chronic amphetamine exposure (an up-regulation in both cases). However, because similar changes were observed in *nad* mutants, these two genes were not present in the reward pool and so are unlikely to account for the non-development of CPP in *nad*.

***nad* mutants highlight the importance of brain developmental transcription factors in the CPP response to amphetamine, and point to a link between amphetamine administration and the control of adult neurogenesis**

An exciting new contribution of our work is to highlight the importance of transcription factors in the response to amphetamine-triggered reward. The category "transcription" was further enriched in the reward pool over individual experiments and so was prominently revealed by our combined microarray strategy. The significance of transcription factors is further strengthened in that all genes classified under "development" in this analysis are also transcription factors (Fig.3B), and is supported by several validations. Firstly, all chosen transcription factors of the reward pool tested by qPCR (n=6) displayed changes in transcription levels upon amphetamine administration in wild-type animals and in four cases these changes were severe enough to be detected by in situ hybridization. The altered response of these 4 genes upon drug treatment in mutants compared to wild-type was also validated by qPCR. Secondly, several of these genes (*Ahr1*, *Dlx1*, *Foxg1*, *Hes5*, *Sox9* and *Tbr1*) have been related to drug use or administration in mammals in other studies (Kerns et al., 2005; Ma, 2007; Mayfield et al., 2002; Sokolov et al., 2003a; Thibault et al., 2000; Treadwell and Singh, 2004). Finally, these genes appear to be functionally connected via the ZFISHDB software, thus they may participate in a common regulatory network. Strikingly, all these genes have recognized roles during vertebrate brain development and also display persistent expression in the adult brain (Suppl. Fig.1) including the mouse brain (see below), suggesting that their relevance for reward-induced behavior could be extended to adult mammals.

Together, these observations suggest that the development of CPP behavior re-uses developmental genes that may possibly maintain a modulatory role during adulthood, perhaps contributing to brain plasticity. Brain plasticity is thought to contribute to the learning of addictive behaviors and can underlie long-lasting changes mediating the persistent effects of addiction (Jones and Bonci, 2005), and several recent reports show that embryonic factors can be recycled in the adult to regulate brain plasticity (e.g. (Sugiyama et al., 2008)). Thus it is possible that the transcription factors identified in our reward pool are normally reused for modulatory or adaptive processes in the adult, and would here contribute to plasticity events triggered by the

drug during the development of CPP. It will be crucial to test the function of these factors in the adult brain, also in the light of behavioral assays.

Classically, the modulatory events believed to underlie addiction involve synaptic or signaling plasticity. The gene regulation network we uncovered can also serve as a valuable entry point towards identifying further plasticity process(es) that might underlie the different behavioral effects of amphetamine in wild-type animals versus *nad* mutants. Our results highlighting developmental transcription factors suggest that fundamental cellular reconfigurations might also contribute to plasticity. In addition, expression of these factors in the mouse and fish brain, and functional assessments in mouse, all point towards a prominent role during neurogenesis. *dlx1a*/*Dlx1* is expressed in the developing mouse ventral forebrain where it controls the formation of GABAergic neurons (Marin et al., 2000). In the adult brain, it is involved in maintaining hippocampal interneurons (Cobos et al., 2005). *Emx1* participates in the regionalization of the embryonic mouse cortex and the production of neuronal subtypes (Gorski et al., 2002; Shinozaki et al., 2002), and adult mice mutant for *Emx1* exhibit impaired hippocampal neurogenesis (Hong et al., 2007). Mouse *Lhx8* is required for the development and maintenance of forebrain cholinergic neurons (Mori et al., 2004; Zhao et al., 2003). Mouse *Sox9* is present in the stem cells of the peripheral and central nervous system, is essential for gliogenesis (Stolt et al., 2003) and has also been isolated as a co-factor for proneural genes (Gohlke et al., 2008). *Tbr1* expression characterizes a freshly postmitotic state in the formation of glutamatergic pyramidal projection neurons of the developing mouse neocortex (Englund et al., 2005), and is maintained during adult hippocampal neurogenesis (Hevner et al., 2006). AhR overexpression in developing neurons has been linked to premature differentiation (Akahoshi et al., 2006). Finally, although Her15 (and its mouse orthologue Hes5) and Foxg1 have not been connected to other transcription factors based on the literature co-citations used by our database, both genes are also expressed in embryonic

neuroepithelial progenitors where they are involved in progenitor maintenance (Hanashima et al., 2004; Hatakeyama et al., 2004; Ohtsuka et al., 1999; Ohtsuka et al., 2001). Later *FoxG1* is strongly expressed in areas of adult neurogenesis, including the subependymal zone of the lateral ventricle and the dentate gyrus (DG) of the hippocampus and juvenile mice haploinsufficient for *FoxG1* show impaired hippocampal neurogenesis (Shen et al., 2006). *Hes5* expression has also been described in astrocytes in neurogenic zones of the adult mouse brain (Ohtsuka et al., 2005). Together, these data suggest that most of the transcription factors recovered in the reward pool are linked by their function or at least their expression at one or the other step of neurogenesis control, including in the adult brain. Although we do not yet have a complete account of zebrafish gene expression patterns at the single cell level, our observations are in agreement with this hypothesis in adult fish as well: the expression of all genes have in common to cover all or part of the adult forebrain ventricular zone (Suppl. Fig.1), which has been demonstrated to be neurogenic (Adolf et al., 2006; Chapouton et al., 2007; Grandel et al., 2006; Pellegrini et al., 2007; Zupanc et al., 2005). The expression profiles of *her15* and *gfi1b* are particularly striking due to their strict restriction to the ventricular zone (Suppl. Fig.1E,F) and their massive down-regulation upon chronic amphetamine treatment (Fig.5A-D). *emx1*, *sox9a* and *tbr1*, are also noteworthy for their prominent expression in the neurogenic area of the lateral pallium (Suppl. Fig.1C,I –arrows- and not shown), an area thought to be the functional equivalent of the hippocampus (Broglio et al., 2005; Salas et al., 2006).

A link between adult neurogenesis and drug abuse has been previously investigated, although with mixed results. Overall, the effect of amphetamine on proliferation during chronic application remains to be examined, although chronic cocaine use has been shown to decrease cell proliferation in the germinal zone of the adult mouse hippocampus ((Dominguez-Escriba et al., 2006; Eisch and Harburg, 2006), for reviews see (Eisch and Harburg, 2006; Venkatesan et al.,

2007)), while withdrawal from cocaine self-administration triggers accelerated maturation of adult newborn hippocampal neurons (Noonan et al., 2008). Given the postulated function of adult hippocampal neurogenesis in the acquisition and consolidation of memories (including their spatial and contextual components), these alterations could play a role in the cognitive processes associated with the development, reinforcement or relapse of addiction. Our results strongly suggest that amphetamine also triggers changes in adult neurogenesis (this paper, and K.W., unpublished), which might involve or result in the changes in transcription factor expression that we observed. It is now important to investigate this point in detail. Our experimental strategy relying on the lack of behavioral response of *nad* mutants further stresses that the regulation of these transcription factors might directly or indirectly link amphetamine and behavior. However, it seems unlikely that the development of CPP observed after 7 days, and which fails in *nad*, could already result from an effect of amphetamine on adult neurogenesis. Newborn neurons require at least 3 weeks to be incorporated into active circuits in the adult mouse and our previous data suggest a similar time-frame in zebrafish (Adolf et al., 2006). It is possible, however, that rapid alterations of the ventricular zone by amphetamine could indirectly affect the physiology of neurons in the vicinity, for instance by altering the trophic support normally provided by ventricular radial glia cells leading to an effect on CPP. These changes could be modified in *nad*. Alternatively, modified neurogenesis upon amphetamine administration could account for later behavioral changes, a hypothesis which remains to be tested in our mutants.

Conclusions

Our experimental strategy based on the *nad* mutant, which fails to respond to amphetamine in the CPP test, allowed the first identification of a subset of amphetamine-regulated transcripts linked to the reward response. This pool contains gene categories previously linked to the use of addictive drugs, thereby validating our data. Enrichment analyses, confirmed by qPCR and in situ

hybridization, highlighted a set of genes encoding transcription factors within this pool, most of which are involved in brain development, and which can partially be organized into a network of functional interactions. Together, we propose that the re-use of a developmental transcription factor-mediated network accompanies or underlies the behavioral response to amphetamine in the adult brain. Some of these factors, expressed in adult neurogenic domains and dramatically down-regulated by amphetamine, can further serve as valuable new entry points into studying the link between neurogenesis and addiction.

Materials and Methods

Animals and maintenance

Adult zebrafish were kept in the fish facility as described in Kimmel et al. (Kimmel et al., 1995). For practical reasons (ease of intraperitoneal injections) all experiments were performed on 5-7 month-old females. In preliminary experiments, we did not notice any difference in the response of males and females to D-amphetamine, for a given genotype (Ninkovic and Bally-Cuif, 2006). Throughout the experiment, care was taken to perform procedures involving animals, such place preference measurements and injections at the same time of the day. Mutagenesis, mutant screening and array experiments involving mutants and siblings were performed on fish of the AB background. Mutant fish were maintained in this background throughout the study. Behavioral experiments on wild-type fish were conducted on an intercross background between AB and Tübingen (Tü). AB, Tü and ABxTü fish have previously been shown to exhibit a clear place preference change in response to amphetamine (Ninkovic and Bally-Cuif, 2006).

ENU mutagenesis and screening for dominant mutations affecting reward

Adult males of the AB strain were subject, over a four-week interval, to four one-hour incubations in 3 mM ENU. Three weeks after this treatment, F1 animals were generated by pair wise mating of ENU mutagenized males with AB females. The specific locus rate at this stage was estimated to be 1/670 against the *golden* (*slc24a5*) locus. Three to nine month-old F1 animals were screened for their change in place preference in response to 40 μg/g D-amphetamine (throughout the text referred to as amphetamine), as described below. Of the 396 F1 adults that were screened, 4 failed to respond to amphetamine, although they exhibited normal place preference without drug and hence could recognize the visual cues of the test tank. They also displayed normal amphetamine content in the brain after injection, as measured using dHPLC (not shown). These animals were considered potential dominant amphetamine-resistant mutants and were crossed against wild-type AB fish to test for transmission of the phenotype. The behavior of 20 F2 adults from these crosses was again assessed in the conditioned place preference test in response to amphetamine. For one of these four F1 candidate mutants, 50% non-responders were obtained in the F2 and all further generations, arguing for a bona fide dominant mutation. We refer to this mutation as $nad^{dne3256}$.

Behavioral assays

The conditioned place preference experiment was performed according to Ninkovic and Bally-Cuif (2006) (Ninkovic and Bally-Cuif, 2006). Briefly, the fish were habituated to a biased two-part chamber (days 1-2), followed by the determination of the initial place preference (day 3). Subsequently, for test animals, amphetamine injections (40 μM) (days 4, 6 and 8) were paired with the initially non-preferred side of the chamber, and control injections of saline solution (days 5 and 7) were paired with the initially preferred side. Control animals are injected with saline every day but likewise paired with the initially non-preferred side on days 4, 6 and 8 and with the initially preferred side on days 5 and 7. On day 9 the final place preference (PP) was measured.

Conditioning was estimated as in Ninkovic and Bally-Cuif (Ninkovic and Bally-Cuif, 2006) as the change in PP before and after treatment, relative to the place preference before treatment. Within a mutant family, fish were designated as mutant (mut), when there was no change or a negative change in place preference after amphetamine administration. Fish were designated as wild-type siblings (sib) when the percentage of change was higher than 5%. If the percentage of change was between 0 and 5% the fish were not included in the microarray analysis, in order to avoid incorrect genotyping.

RNA extraction and microarray study design

One-color microarray experiments were performed using 3 replicates for each condition. Each replicate contained the RNA from 4-5 pooled brains. To prepare these samples, total RNA was extracted from whole brains using RNeasy mini kit (Qiagen), following the protocol "Purification of Total RNA from Animal Tissues". For the disruption and homogenization step, brains were dissected and immediately frozen in liquid nitrogen. Then, 600 µl buffer RLT was added to each brain and the tissue was homogenized using a needle and syringe. The samples were individually controlled for RNA quality and genomic contamination using 2100 Bioanalyzer (Agilent technologies), according to the manufacturer's instructions. Samples from 4-5 brains were then pooled to generate a single replicate. The animals used in the different experiments were all aged between 6 and 12 months and were manipulated as follows: (i) Experiment 1: wt+: AB fish subject to the CPP behavioral assay and sacrificed immediately after the final place preference measurement (day 9); wt-: AB control fish of the CPP behavioral assay; (ii) Experiment 2: mut+: AB animals from a *nad* family of the F6 generation (obtained from pairing a *nad*/+ F5 heterozygote fish and an AB fish) identified as mutant based on the CPP assay and sacrificed immediately after the final measurement on day 9; mut-: siblings identified as wild-type based on the CPP assay in the same experiment; (iii) Experiment 3: mut-: AB animals from a F5 *nad*

family identified as mutant in the CPP test and left without drug for 2 months afterwards; sib-: siblings identified as wild-type in the same experiment and left without drug for 2 months.

RNA Amplification, Labeling and Hybridization

The RNA samples were amplified with the Agilent Low Input Linear Amplification kit PLUS, One Color (Agilent Technologies, Palo Alto, CA). The labeling, hybridization and data extraction were performed at ServiceXS (Leiden, The Netherlands). Briefly, 500 ng total RNA in an 8.3 µl volume was mixed with 1.2 µl of T7 promoter primer. Primer and template were denatured by incubating at 65°C for 10 minutes and annealed by placing the reaction on ice. The First Strand Reaction was performed by adding a master mix containing 5x First Strand Buffer, DTT, 10 mM dNTP mix, RNaseOUT, and Moloney murine leukemia virus reverse transcriptase, and incubated at 40°C for 2 hours. MMLV-RT enzyme was inactivated by incubation at 65°C for 15 minutes and the samples were directly transferred to ice. Samples were labeled by adding 2.4 µl cyanine 3-CTP. *In vitro* transcription was initiated by addition of the IVT Mastermix containing 4x transcription buffer, DTT, NTP mix, 50% PEG, RNaseOUT, Inorganic Pyrophosphates, T7 RNA polymerase and incubated at 40°C for 2 hours. Qiagen RNeasy mini spin columns were used for purification of the labeled cRNA as described in the Agilent user manual. After amplification and purification, the samples were checked for RNA concentration and dye incorporation on the Nanodrop ND-1000 by using one µl of the 60 µl elution solution (nuclease-free water). Hybridization and washing was performed using the standard Agilent protocol.

The microarray slides were custom designed by Agilent Technologies. The slides contained in total 43 371 probes of a 60 oligonucleotide length. Of these probes a total of 21 496 probes were identical to the probes present on the Agilent probe set that is commercially available under

catalog number 013223_D. Most of the additional probes were designed using the eArray software from Agilent Technologies (https://earray.chem.agilent.com/earray/). Settings used were the following: base composition methodology, best probe methodology and design with 3' bias. The Agilent *D.rerio* transcriptome was used as a reference database. A small number of probes were manually designed in order to obtain gene-specific probes for members of larger gene families. The complete design of the microarrays has been submitted to the GEO database, under the platform submission number GPL7735.

Microarray Imaging and Data analysis.

Scanning of the microarray slides was performed using the Agilent dual laser DNA microarray scanner. The microarray data were processed from raw data image files with Feature Extraction Software v9.1 software, protocol GE1-v1_91(Agilent Technologies, Palo Alto, CA). Processed data were subsequently imported into Rosetta Resolver 7.1 (Rosetta Biosoftware, Seattle, Washington) and subjected to default intensity error modeling. Results from triplicate experiments were combined using the default intensity experiment builder. Ratio experiments were built from the intensity data using the Agilent/Intensity-pair wise ratio builder with the control group (salt/wild type) as baseline. Data were analyzed at the level of UniGene clusters (UniGene build #105). Unigene list of individual experiments can be found on Supp. Tables 3-5. The significance cut-offs were set at $P \leq 0.01$ and absolute fold change ≥ 1.5. Venn diagrams were constructed using the compare biosets function of Rosetta resolver. All microarray data were submitted to the GEO database (currently under revision).

Gene ontology (GO) term enrichment analysis

GO term enrichment analysis was performed on differentially expressed genes ($p<0.01$; fold change <-1.5 or >1.5) from the individual experiments, as well as on the reward pool. Lists of

differentially expressed genes were imported into Pathway Studio (Ariadne Genomics, Rockville, USA). Before analysis the ResNet 5.0 database of this software was extended to include the zebrafish protein annotation. The Pathway Studio program determines the human, rat and mouse orthologues of the zebrafish transcripts, using the BLAST best reciprocal hit method (Ispolatov et al., 2005). This information is used to perform the enrichment analysis, in which Pathway Studio calculates the statistical significance of the overlap between the input list and a GO group by applying Fisher's exact test. The resulting p-value depends on the extent of overlap between the input list and a group as well as the sizes of the list and a group. We considered a GO term to be significantly enriched if $p<0.01$.

Assessment of the functional interactions between recovered genes

Functional interactions between zebrafish genes in the reward pool were inferred from the STRING database (von Mering et al., 2007). STRING integrates and scores information derived from high-throughput experiments, genomic context, and previous knowledge like text-mining of abstracts. For zebrafish, the number of interactions is small compared to better annotated species like mouse or human. In order to enrich the interactions in our gene set, we transferred interactions from orthologous genes as provided by the COG-mode of STRING, where the information of orthologous groups of proteins relies on an extended version of the COG database (Tatusov et al., 2003). The ZebraFish Interaction SearcH DataBase (ZFISHDB) integrates all interactions between clusters of orthologous genes, relying on STRING 7.0. It allows the input of a set of fish genes and outputs interactions between those genes with a STRING combined score above 0.8. In addition, ZFISHDB offers a gene ontology filter to reduce the size of large data sets. It is publicly available at http://mips.helmholtz-muenchen.de/zfishdb/. Genes without mouse homologues are not considered by the present version of the database.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from whole brains using the RNeasy mini kit (Qiagen). The qPCR experiments and the statistical analysis were performed using the LightCycler 1.2 system (Roche) and the relative expression software tool REST (Pfaffl et al., 2002) as previously described (Leucht et al., 2008). Real time PCR experiments were performed in replicates of 8. The list of used primers and probes is provided in Suppl. Table 6.

In situ hybridization

In situ hybridization (ISH) was performed on 5-6 month-old AB/Tü fish, which had either been treated with amphetamine (40 μM) or saline solution, once a day, for 18 days. Animals were sacrificed and the brains were removed after fixation in 4% paraformaldehyde. Dissected brains were then postfixed in 4% paraformaldehyde overnight. The brains were then embedded in albumin-gelatine:sucrose denatured with glutaraldehyde. Cross sections of 70 μm were made using a vibratome, after which the sections were washed in PBT and dehydrated through a methanol series. In situ hybridization was performed according to published protocols (Hauptmann and Gerster, 1994) for whole-mount embryos, followed by staining for alkaline phosphatase activity using NBT-BCIP. Initially one brain was used per treatment. All sections were photographed and corresponding sections were compared between treatments. The in situ hybridization for genes, the expression patterns of which showed a visible difference between amphetamine treatment and control, was repeated once. Sections were photographed with an Axioplan2 stereomicroscope and processed using the Axiovision 4.1 software (Zeiss). To generate probes, partial cDNAs for the genes of interest were cloned from PCR products (PCR conditions available upon request, PCR primers provided in Suppl. Table 7) into pCRII-TOPO (Invitrogen) (for *her15*) or pSC-A-amp/kan using the StrataClone PCR Cloning Kit (Stratagene) (for all other genes) following the manufacturer's instructions. All clones were verified by

sequencing. The RNA probes were synthesized following published protocols (Hauptmann and Gerster, 1994).

List of abbreviations used

Conditioned Place Preference CPP

Authors' contributions

K.J.W. designed and performed the experiments and wrote the manuscript (with L.B-C.), W.N. performed mutant identification experiments and worked on the manuscript, J.N. conducted the behavior screen and identified the *nad* mutant, S.T. provided technical assistance for in situ hybridizations, D.T. performed the Pathway Studio analyses in W.W.'s lab, A.H.M. and H.P.S. designed the arrays and helped with their interpretation, D.H., C.M. and F.T. designed the ZFISHDB database, L.B-C. directed the work, designed the experiments and wrote the manuscript (with K.J.W.).

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Legends to Figures and Tables

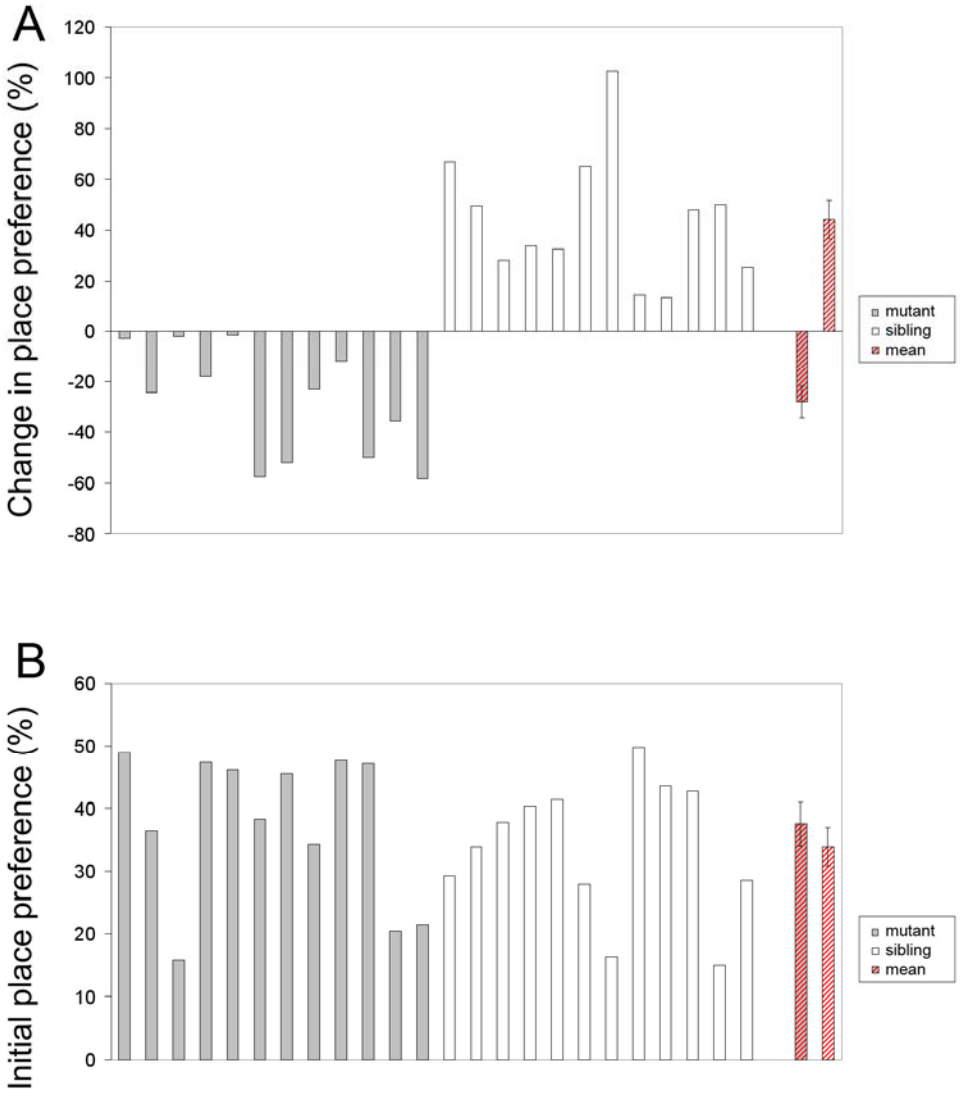
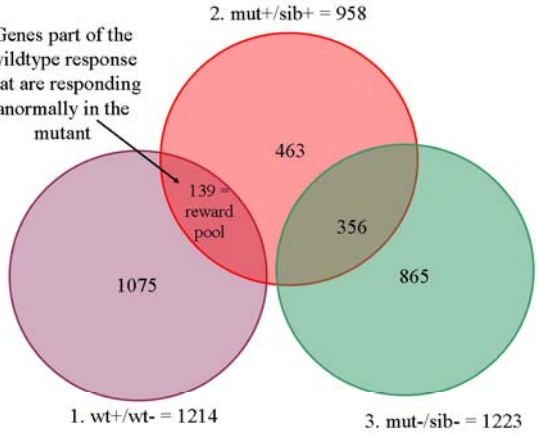


Figure 1. The dominant mutant *nad*^{dne3256} shows no response to amphetamine, but a normal initial place preference. **A)** Conditioned place preference (%) of 24 individuals of a *nad*^{dne3256} family (generation F3), showing 12 mutants and 12 siblings. Mutants were defined as showing no, or a negative change in place preference. Siblings were defined as having a change in place preference of 5% or over. The last two bars represent the means for both groups. The difference between the means is statically significant (T-test with unequal variances; p=2.3E-07). **B)** Initial place preference (%) for the same 24 individual fish. The last two bars represent the means for both groups. There is no significant difference between the two means (two sample unequal variance t-test p=0.45). Error bars represent the one fold of the standard error.

A

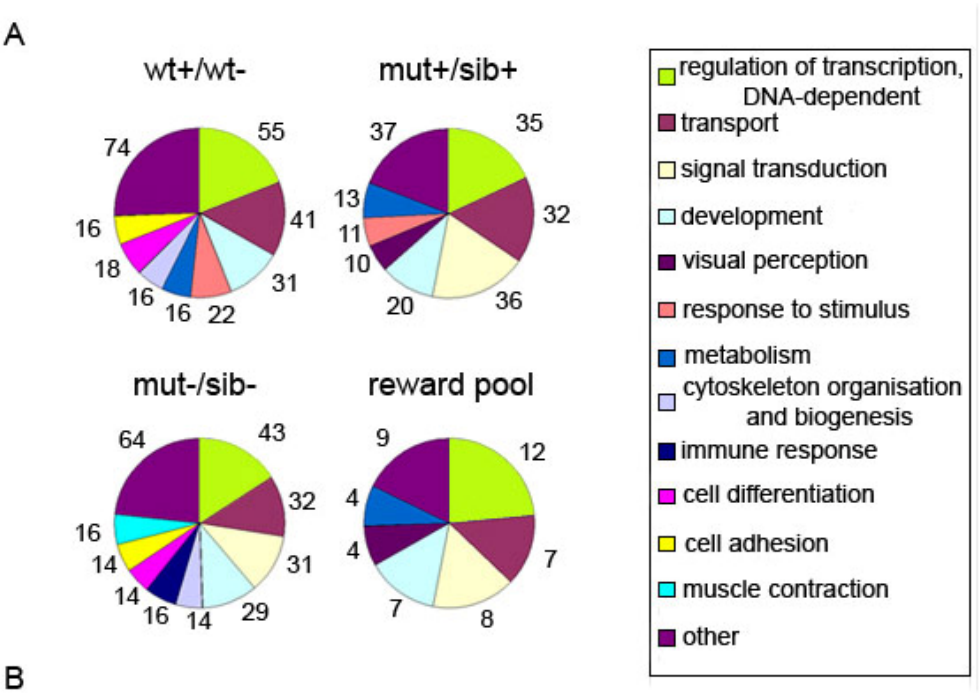


B

Direction of differential expression in wt+ (vs. wt -)	Direction of differential expression in mut+ (vs. sib+)	Conclusion	No. of transcripts (%)
↑	↑	Increased up-regulation in mutant	24 (17)
↓	↓	Increased down-regulation in mutant	33 (24)
↓	↑	Mutant fails to downregulate	33 (24)
↑	↓	Mutant fails to upregulate	49 (35)

Figure 2. 'Reward pool' genes characterize the transcriptional response to amphetamine-triggered CPP. A) Diagram of differentially expressed genes from microarray experiments. Individual microarray experiments were combined to reveal a reward pool. A comparison of the differential expression from two experiments showed no bias in the direction of expression. Pool 1 shows the genes differentially expressed in "wild-type with amphetamine vs. wild-type without amphetamine". Pool 2 represents genes differentially expressed in "mutant with amphetamine vs. sibling with amphetamine". Pool 3 represents genes differentially expressed in "mutant without amphetamine vs. non-mutant siblings without amphetamine". The genes in Pool 3 were subtracted from Pool 2, in order to eliminate basal differences between mutants and siblings, not due to amphetamine administration. The intersection of the remaining genes in pool 2 and the genes in pool 1 forms the "reward pool". The genes in this pool are differentially expressed in both experiments – that is, they are involved in the wild-type response to amphetamine, as well as the non-response to amphetamine in the mutant. **B)** Comparison of the direction of regulation (up- or down-) of transcripts from the reward pool for the experiments wt+/wt- and mut+/sib+. No bias towards a particular pattern can be observed.

A



B

GO term	Protein
regulation of transcription, DNA-dependent	Her15, Ahr1a, Sox9a, Dlx4a, HBP17-I, Gfi1b, Dlx1a, Hoxd12a, Foxg1, Emx1, tbr1, Lhx8
development	Her15, Dlx4a, Dlx1a, Hoxd12a, Foxg1, Emx1
visual perception	GJA8-I, Tacstd, Rbp2a/2b, Gnat2
transport	KCNS1-I, Crat, GJA8-I, Slc6a5, Rbp2a/2b, Chrne, Fabp10, Atp1a1a.3, Atp1a1a.4, Pdzk1I, ENAH-I, DKEY-1J5.1-I (enabled homologue)
metabolism	Slc6a5, OXCT2-I, Atp1a1a.3, Atp1a1a.4
signal transduction and cell-cell signaling	ADM-I, Tsc2, Ahr1a, HBP17-I, Anxa3, Chrne, Gnat2, ZA20D3-I, GJA8-I

Figure 3. Categorization of the 139 genes contained in the reward pool. A) Pie charts showing superordinate categories of significantly enriched gene ontology (GO) terms for the three individual experiments as well as the reward pool. Numbers indicate the number of transcripts assigned to a particular category. Categories containing less than 5% of the total number of genes were classified as "others". **B)** Gene ontology (GO) terms for biological process significantly enriched ($p<0.01$; Pathway Studio) in the reward pool, with their corresponding Proteins. Of the 139 genes in the reward pool, 31 have been annotated and can be found in the ZebraFish Information Network (www.zfin.org). Our manual annotation identified related mammalian genes for 84 genes (see Discussion). Genes chosen for validation are labeled in red.

Appendix 8.3. Zebrafish reward mutants reveal novel transcripts mediating the behavioural effects of amphetamine

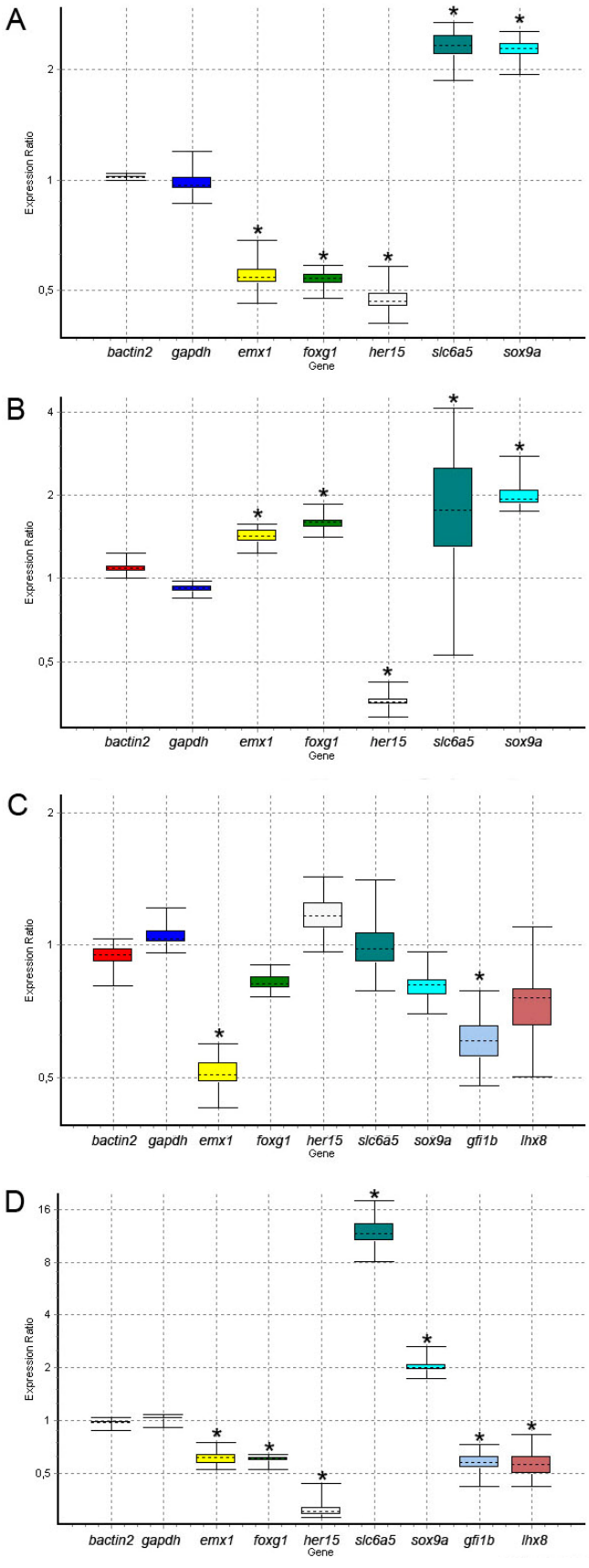


Figure 4 (see above). Validation and categorization of transcripts in acute and /or chronic response to amphetamine using quantitative PCR. Individual genes with different biological roles were selected from the reward pool (see Fig. 3B) for qPCR using the original RNA from wt+/wt- **(A)** and mut+/sib+ **(B)**. The qPCR experiment revealed selected genes showed expression changes similar to those seen in the microarray results. qPCR was also performed on the brain of fish injected with 1 dose (acute) or 18 doses of amphetamine (chronic). Two genes, *gfi1b* and *emx1*, were downregulated after one dose of amphetamine **(C)**. The remaining transcripts were down or up-regulated in the same direction as the microarray in the chronic situation **(D)**. Boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. * significant using REST software.

Appendix 8.3. Zebrafish reward mutants reveal novel transcripts mediating the behavioural effects of amphetamine

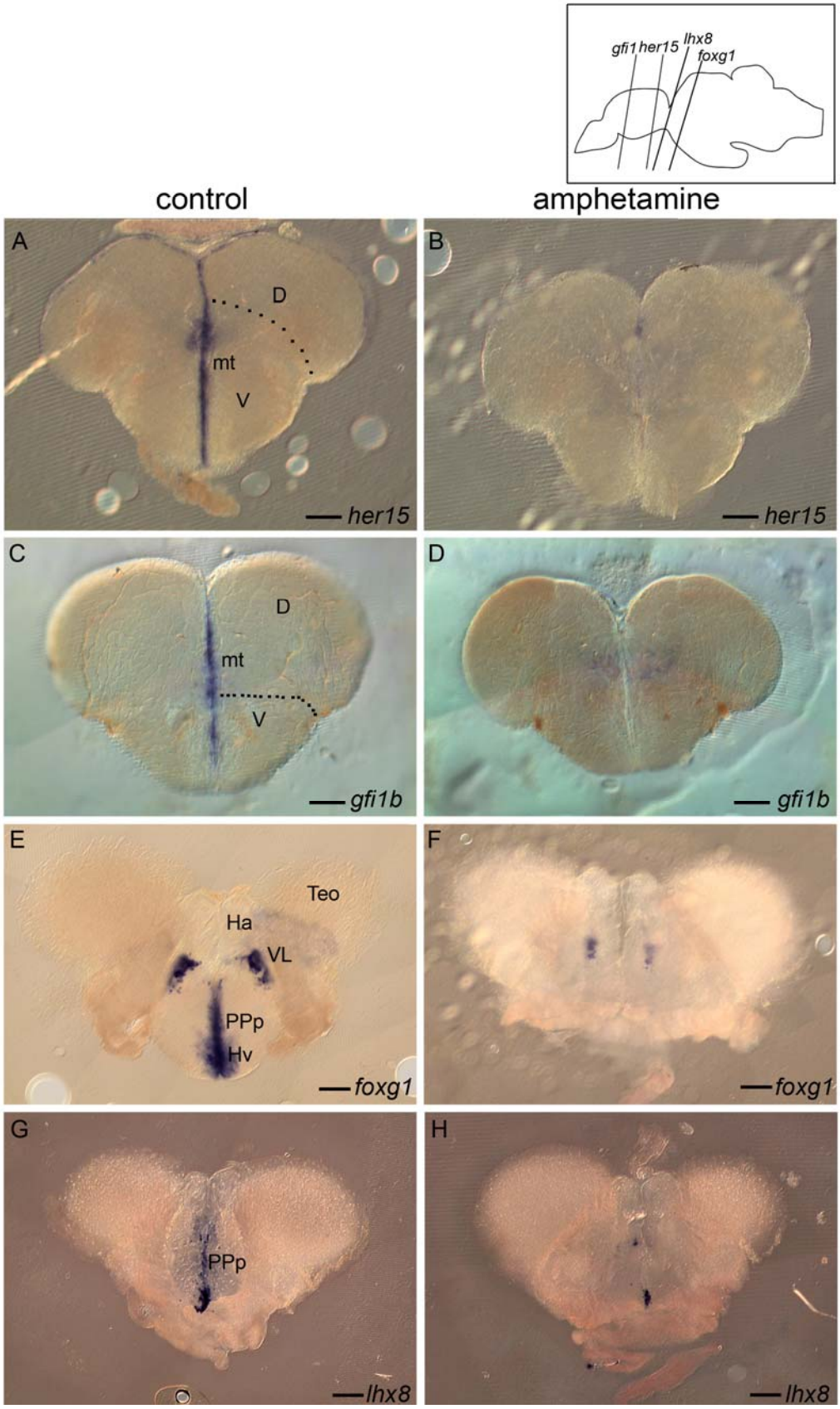


Figure 5 (see above). Candidate genes validated using in situ hybridization. *gfi1b* and *her15* are expressed in ventricular zones throughout the brain, including in the midline of the telencephalon **(A, C)**. Upon chronic amphetamine administration this expression is visibly down-regulated **(B, D)**, and this throughout the brain (see also Suppl. Fig. 2A-D). Upon amphetamine administration, the expression of *foxg1* and *lhx8* is reduced in the parvocellular preoptic nucleus, posterior part (PPp) (*foxg1* and *lhx8*) **(F, H)** and in the ventral zone of the periventricular hypothalamus (Hv) (*foxg1*), when compared to the brains of animals injected with a saline solution **(E, G)**. The expression pattern of these genes remains unchanged in other brain areas upon amphetamine administration (see Suppl. Fig.2E-H). Scale bars = 100 m in all panels. D = dorsal telencephalic area; V = ventral telencephalic area; mt = midline of the telencephalon; PPp = parvocellular preoptic nucleus, posterior part; Hv = ventral zone of the periventricular hypothalamus ; VL = ventrolateral thalamic nucleus.

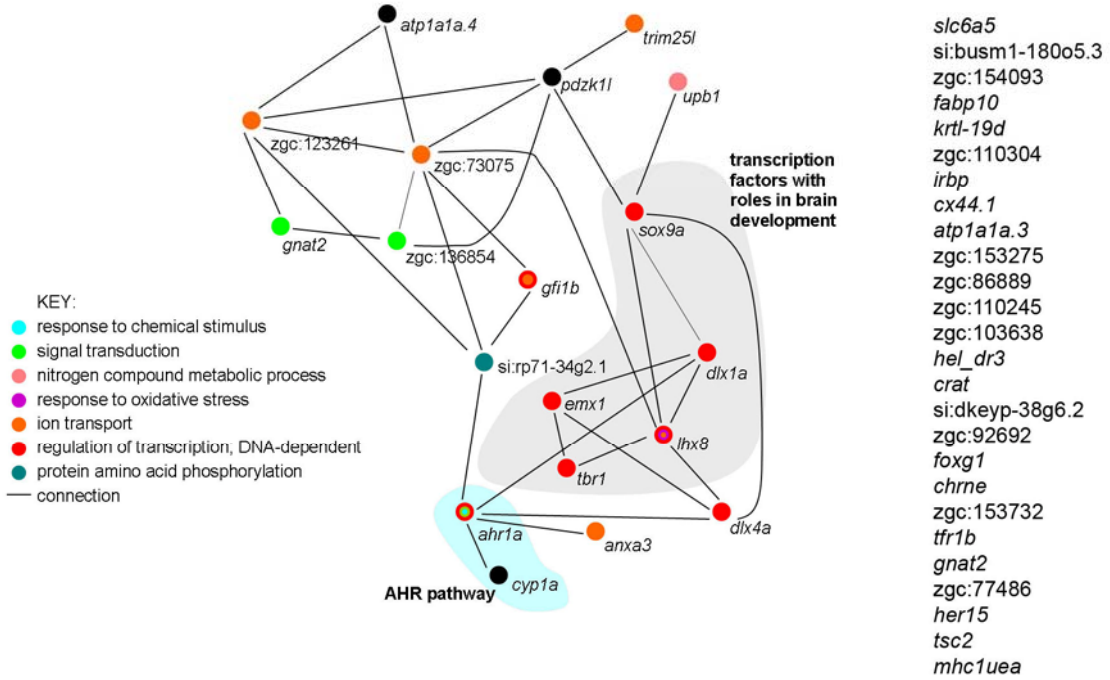
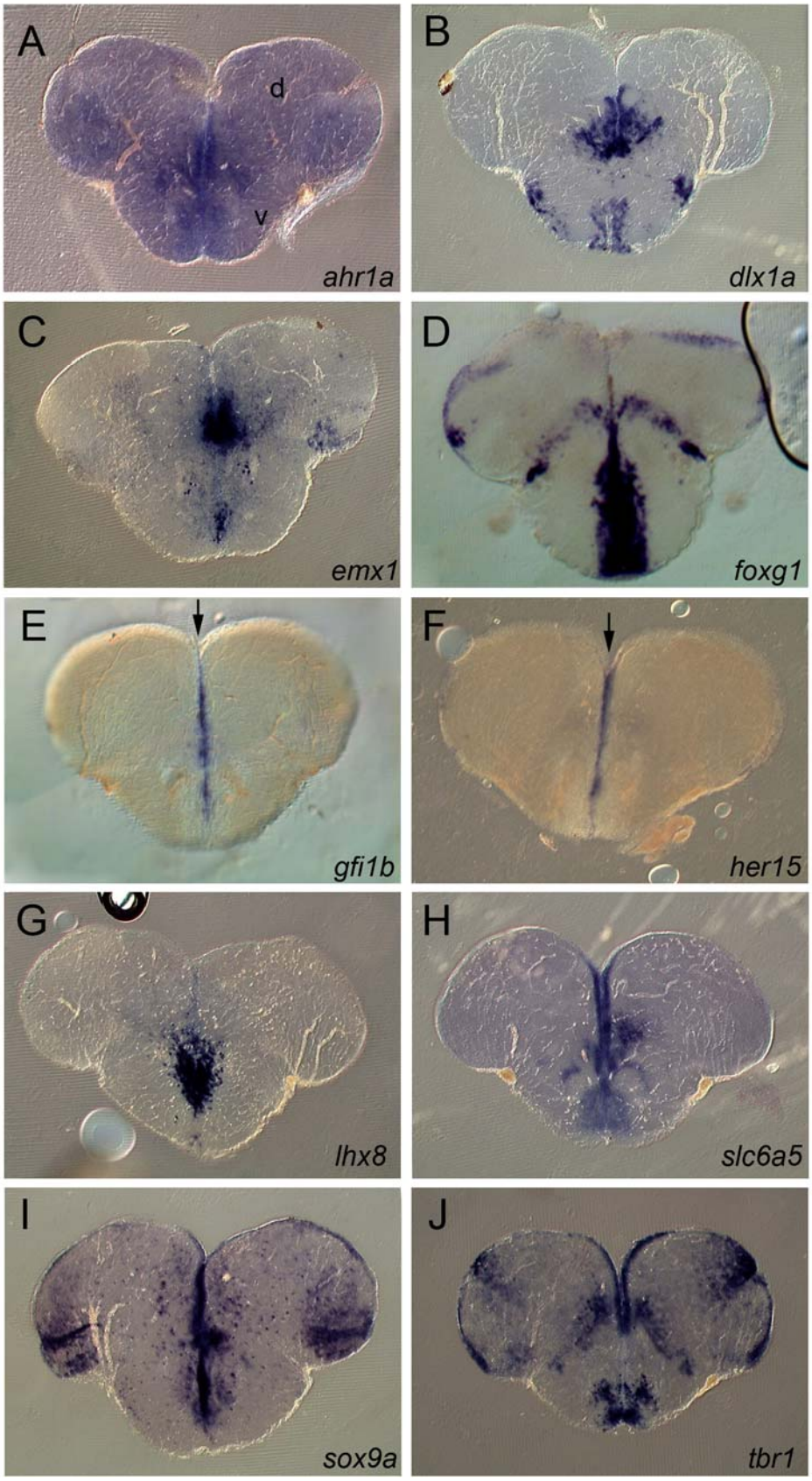


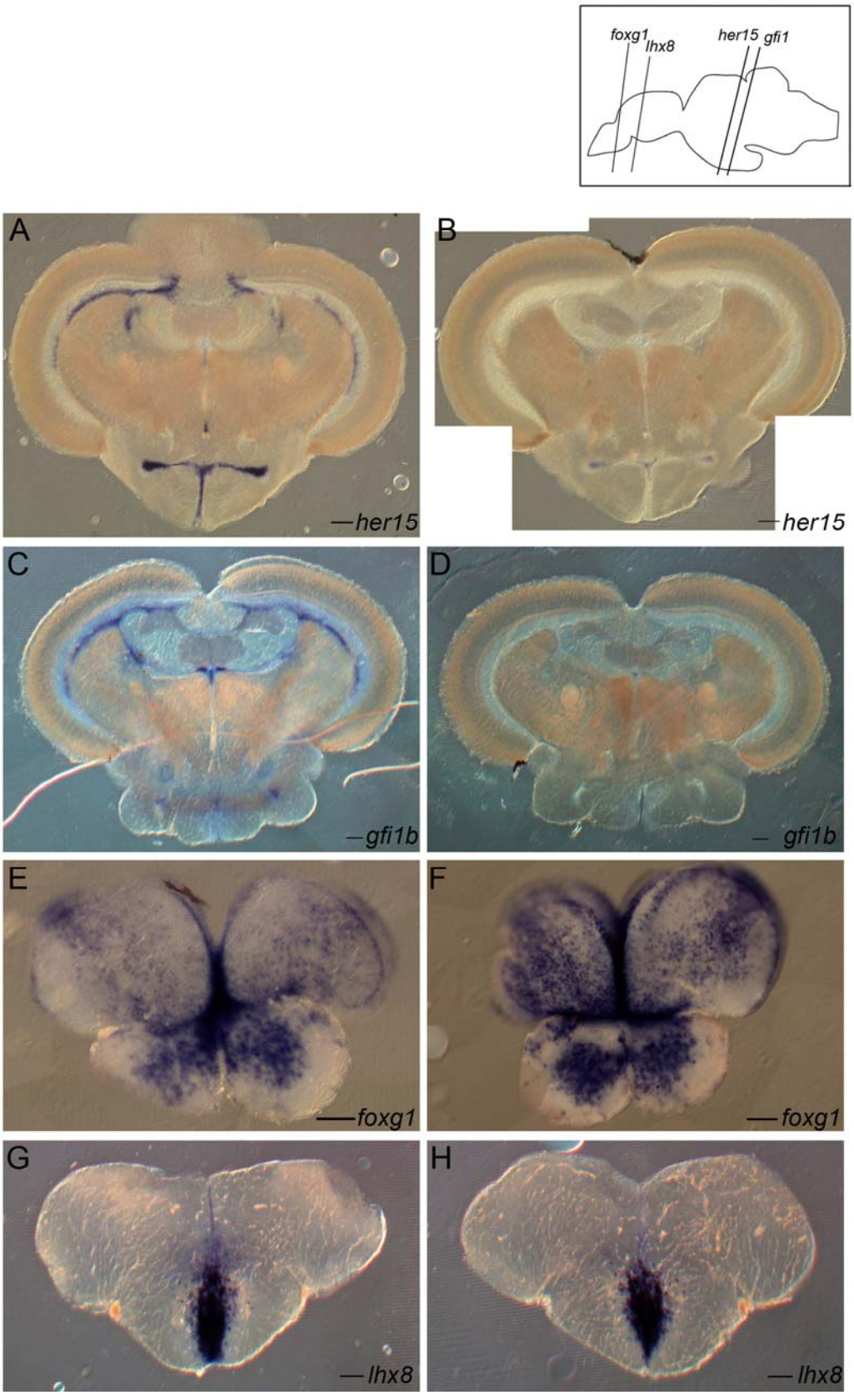
Figure 6. Network view of 18 genes from the reward pool functionally linked by the ZFISHDB database. Nodes are connected if functional interactions between the genes are provided by the Cluster of Orthologous Genes (COG) mode of the STRING database. The GO terms listed are not exhaustive. The genes, which have a mouse homologue and were thus included in the analysis, but which were not linked to other genes in the pool, are listed separately on the right. In addition, *her15*, *tsc2* and *mhc1uea*, for which the program did not find suitable mouse homologues, but for which we manually checked for associates with the other genes in the network, are also listed.

Appendix 8.3. Zebrafish reward mutants reveal novel transcripts mediating the behavioural effects of amphetamine



Supplementary Figure 1 (see above). Expression in the adult brain of the 10 differentially regulated transcripts chosen for validation (in situ hybridization -blue signal- on cross sections at telencephalic levels, dorsal up). *ahr1a* (A), *dlx1a* (B), *emx1* (C), *foxg1* (D), *gfi1b* (E), *her15* (F), *lhx8* (G), *slc6a5* (H), *sox9a* (I) and *tbr1* (J) are expressed in the adult zebrafish brain, including the telencephalon, as illustrated here. *gfi1b* and *her15* are restricted to the ventricular zone (midline, arrows). d=dorsal telencephalic area (pallium); v=ventral telencephalic area (subpallium).

Appendix 8.3. Zebrafish reward mutants reveal novel transcripts mediating the behavioural effects of amphetamine



Supplementary Figure 2 (see above). Expression of *her15*, *gfi1b*, *foxg1* and *lhx8* upon chronic amphetamine administration, as revealed by in situ hybridization. The expression of *her15* and *gfi1b* was lost upon amphetamine administration throughout the brain (A-D) (see also Fig.2A-D). The expression of *foxg1* and *lhx8* were changed in the parvocellular preoptic nucleus (*foxg1* and *lhx8*) and in the ventral zone of the periventricular hypothalamus (*foxg1*) (see Fig.2E-H) but not in other areas of the brain, including the telencephalon (E-H).

Supplementary Table 1. List and manual annotation of the 139 genes contained in the reward pool. All of these transcripts were differentially regulated ($p<0.01$ and fold change <-1.5 or >1.5 (2.d.p)) in both mut+/sib+ and wt+/wt- but not in mut-/sib-. Colors (except in the right column) relate to the respective directions of the fold change in the mut+/sib+ versus wt+/wt- experiments and correspond to the categories defined in Fig.2B. Genes referred to in the text are marked in bold. Where no zebrafish description was available, the human homologue is listed in brackets, along with the human composite Unigene from ENSDARG. Linkage of the zebrafish probes to human Unigenes was done via ENSDARG accession numbers using the Biomart software (http://www.ensembl.org/biomart). Human Unigenes were obtained from the ENSG codes using the Clone/Gen ID converter software (http://idconverter.bioinfo.cnio.es/). In several cases when no ENSDARG identifier was available for the zebrafish probe it was possible to obtain a human Unigene link using the Homologene database (http://www.ncbi.nlm.nih.gov/homologene). "KARG database entry" (right column) refers to a manual search of the Knowledgebase for Addiction Related Genes (Li et al., 2008). The GO terms listed are not exhaustive but correspond to those relevant to addiction (color-coded in the right column).

See accompanying DVD.

Supplementary Table 2. Fold change values for qPCR and microarray experiments. Fold change for the microarray experiments was calculated as described in the methods section. For the qPCR data, fold change was estimated for expression ratios less than 1 (calculated using REST), as the negative reciprocal of fold change. Values greater than one were used as fold change. For the qPCR significant differences in expression are in green ($p<0.01$, REST software); non-significant values are in red. The qPCR showed the similar trends to those observed in the microarray, thus validating our array. For the acute experiment, *emx1* and *gfi1b* were differentially regulated in the same direction as the microarray. The remaining transcripts were not differentially regulated. For the chronic experiment all transcripts followed the pattern observed in the wt+/wt-, indicating that our experimental setup in the microarray identified genes involved in the chronic response to amphetamine. "-": not tested.

Transcript	Acute (x1)	Chronic (x18)	wt+/wt- original RNA	wt+/wt- microarray	mut+/sib+ original RNA	mut+/sib+ microarray
her15	1.16	0.71	-2.08	-100	-3.03	-100
emx1	-1.96	-1.60	-1.59	-1.60	1.30	1.66
foxg1	-1.22	-1.64	-1.85	-1.64	1.46	1.84
lhx8	-1.37	-1.73	-	-1.97	-	1.59
gfi1b	-1.66	-1.27	-	-1.79	-	-1.57
sox9a	0.81	2.09	2.27	1.57	1.83	2.08
slc6a5	0.99	12.24	2.32	1.65	1.69	1.51

Supplementary Table 3. Unigene list of genes differentially expressed between wild-type with amphetamine and wild-type with saline. 1214 genes were found to be differentially expressed between wild-type fish that received amphetamine treatment triggering CPP versus fish that received a control, saline treatment (microarray experiment 1 "wt+/wt-").

See accompanying DVD.

Supplementary Table 4. Unigene list of genes differentially expressed between mutants with amphetamine and siblings with amphetamine. 958 genes were differentially expressed between mutants and their wild-type siblings upon amphetamine treatment (microarray experiment 2, “mut+/sib+”).

See accompanying DVD.

Supplementary Table 5. Unigene list of genes differentially expressed between mutants without treatment and wild-type siblings without treatment. 1224 genes were differentially expressed under the “mut-/sib-” conditions. We took these to represent the basal differences between the mutants and their wild-type siblings (microarray experiment 3, “mut-/sib-”).

See accompanying DVD.

Supplementary Table 6. Primer sequences and probe numbers (Universal Probe Library (Roche)) for quantitative real-time PCR.

Transcript	Forward primer	Reverse primer	Probe (UPL)
emx1	TTGGACATCGGTTTCAAGGT	GAACGGTCCGTGTAGTAGCAG	14
foxg1	TCCGTATTACCGGGAGAACA	AAACTCAAGTTGTGTCTGATG GAA	121
gfi1b	TGAAGAAACACACATTTATCCAC A	TGAATGCTTTTCCACACACC	7
her15	CCAACAAGGAGAAGCACAAAT	GATCCTGCTGCTGGAACTCT	67
lhx8	CAGCGTGTGCCAAACATC	AACGTGTTCCGTATTTCCTGA	113
slc6a5	GCCACTGGCTGCTTGTCT	CGGGAATGTTGCTGTGAAAT	129
sox9a	GTCCAGCATGGGAGAAGTG	TCAGTTTTCGGGGTGGTG	95
bactin2	AAGGCCAACAGGGAAAAGAT	GTGGTACGACCAGAGGCATAC	56
gapdh	AACTTTGGTATTGAGGAGGCTCT	TCTTCTGTGTGGCGGTGTAG	114

Supplementary Table 7. Primer sequences used to clone partial cDNAs serving as ISH probe templates.

Transcript	Forward primer	Reverse primer
ahr1a	CGGCATGAGTTTCAGAGACA	AAGGGGCAGGATCAGAAGAT
dlx1a	GAGAGAGCGAGAGCGAGAGA	GGGGTTGTTTCAGCAGTCCT
emx1	CAGCTGGACTCTTCTGGTCA	TGTGTTCATTTGGGCAGTGT
foxg1	AGGAGTTGCCAGAGCAAGAG	CACGTTGCTGACAGTGGAAT
gfi1b	AGCGGCCTACTCCAACCTAT	CACTTCAGTCCGTGCTGTGT
her15	CGCTCTGCTCAGAGAAACAGC	TCCATGAGGAAAACTACACTA
lhx8	GGCAGCAGCACCTATATGGA	CATGCTGTCCTCTGACCTGA
slc6a5	AACACCAAACCGGAGAACAG	CTCGCTAGGGCTGACATAGG
sox9a	CCGTGGATTTGCAGGAATTA	CCCAATGCATCATGATTTTTC
tbr1	TCTACACAGGCTGCGACATC	CCTTGGAGCAGTTTTTCTCG

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10. Curriculum vitae

Lebenslauf
Katharine Joy Webb

Geboren in London am 15. März 1979
Bildungsweg:

1999-2002	Bachelor of Science (Hauptfach: Zoologie) von der University of Otago, Dunedin, Neuseeland
2002-2005	Biologie-Diplom an der Universität Konstanz, BRD
2004	Diplomprüfung in Tierphysiologie und Entwicklungsbiologie (Note: 1,0)
2005	Diplomarbeit im Labor von Professor Axel Meyer/Dr. Gerrit Begemann: "The role of N-terminal acetyltransferases in embryonic development" (Note: 1,3)
April 2005-	Doktorandin im Labor von Dr. Laure Bally-Cuif, GSF, München, BRD: "Neurogenesis control in vertebrates: molecular and cellular characterization of a progenitor pool at the midbrain-hindbrain boundary in the zebrafish embryo"

Presentationen auf Internationalen Konferenzen:

Mai 2007	9th Annual Meeting of the International Behavioural and Neural Genetics Society (IBANGS) (Doorwerth, The Netherlands): Vortragstitel: "Identification of mutations affecting amphetamine-induced reward in zebrafish"
März 2008	5th ZF-Models consortium meeting, Paris 29th to 30th March 2008. Vortragstitel: "Identification of the genetic networks underlying addiction behaviour using zebrafish."
Juli 2008	8th Conference on Zebrafish Development and Genetics, Madison, USA. Vortragstitel: "Identification of the Genetic Networks underlying *Amphetamine*-induced Reward in *Zebrafish.*"

Kürzlich belegte Kurse und Fortbildungen:

März 2006	"Transcriptomics and Proteomics in Zebrafish", Lorentz Centre Leiden, Niederlande
April 2006	"2 day Gene Expression Training", Agilent Technologies, Genomics DemoLab Waldbronn, BRD

2006-	Eingeschrieben im "Bioinformatics online postgraduate certificate programme", der University of Leeds und der University of Manchester, Grossbritannien
	Abgeschlossene Kursmodule (jeweils ein Semester):
	- Introduction to Bioinformatics: Use of programs for sequence analysis - Introduction to software development in Java
März 2007	“7th Course in Bioinformatics”, European School of Genetic Medicine, Bertinoro di Romagna, Italien