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GENEESKUNDE

master in de biomedische wetenschappen: milieu en gezondheid

Masterproef

Short and long range signalling during brain regeneration in the planarian Schmidtea mediterranea and the involvement of the nou-darake (ndk) genes

Promotor :
dr. Karen SMEETS

Promotor :
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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting milieu en gezondheid

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Acknowledgements

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Abbreviations

AP	Anterior-posterior
CNS	Central nervous system
dsRNA	Double-stranded RNA
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FGFRL	Fibroblast growth factor receptor-like
GJ	Gap junction
GJC	Gap junction communication
Gli	Gliotactin
Hh	Hedgehog
HU	Hydroxyurea
Ig	Immunoglobulin
Ndk	Nou-darake
Ptc	Patched
RNAi	RNA interference
RT	Room temperature
Smo	Smoothened
VNC	Ventral nerve cord

Abstract

Planarians are becoming increasingly commonly used as model organisms to study regeneration. These animals posses a large population of adult stem-cells termed neoblasts, which underly their abilitity to regenerate a complete organism from just a small fragment of tissue. A complex system of pathways is necessary to control this regeneration ability and maintain correct patterning. NDK, a transmembrane protein similar to FGFR, is involved in the regulation of brain regeneration. We identified four homologues of *ndk* in the planarian *Schmidtea mediterranea*. One of these *ndk* homologues is unreported in the scientific literature and we named this gene product *ndk2*. Not only short range, but also gap-junctional (GJ) communication plays a crucial role in correct patterning. The GJ-signalling is studied in three planarian species: *D. japonica, S. mediterranea* and *G. tigrina*.

In situ hybridizations were used to study the expression patterns of the *ndk* genes. *Ndk* and *ndk2* are expressed in very similar patterns in intact and regenerating animals, showing expression of both genes in the brains and pharynx. Interestingly, all *ndk* genes are expressed in the pharynges, including *ndk3* and *ndk4*. *Ndk(RNAi)* results in the formation of early ectopic brain ganglia and posterior brain expansion. In contrast to *ndk(RNAi),* inhibitions of the other *ndk* genes do not cause phenotypic malformations. The roles of these genes remain unknown and their expression in the pharynx might provide crucial information about their functioning in the planarian *Schmidtea mediterranea.* Anti-phospho histone H3 immunostainings were performed to study possible roles of *ndk* on stem cell proliferation. Small increases in the number of mitoses were observed in intact and regenerating *ndk(RNAi)* animals.

Gap junctional communication was investigated by using octanol treatment in three planarian species: *D. japonica, S. mediterranea* and *G. tigrina*. Bipolar head formation after anterior and posterior amputations is only observed in *D. japonica*, revealing species-specific effects of octanol treatment on the regeneration of planarians.

Only a few answers have been revealed and additional research is necessary to obtain crucial information about the short and long range signalling during brain regeneration, and the involvement of the *nou-darake* genes in planarians.

Samenvatting

Planaria zijn veelgebruikte modelorganismen om het regeneratieve proces te bestuderen. Door het bezit van een populatie totipotente adulte stamcellen, genaamd neoblasten, beschikken deze platwormen over de mogelijkheid om een volledig organisme te regenereren uit slechts enkele cellen. Tijdens de regeneratie dient een correcte polariteit behouden te worden. NDK, een transmembranair proteïne gelijkend op FGFR, reguleert de hersenregeneratie in planaria. In dit project werden vier homologen van *ndk* geïdentificeerd in de platworm *Schmidtea mediterranea*. Eén van deze genen is onbekend in de wetenschappelijke literatuur en werd *ndk2* genoemd. Ook communicatie via gap junctions (GJ) speelt een belangrijke rol in het behoud van een correcte polariteit. GJ-signalering werd bestudeerd in drie planaria soorten: *D. japonica, S. mediterranea* and *G. tigrina*.

In situ hybridizaties werden uitgevoerd om de expressiepatronen van de *ndk* genen te bestuderen. *Ndk* en *ndk2* vertonen zeer gelijkaardige expressiepatronen in zowel intact als regenererende organismen, met expressie van beide genen in de hersenganglia en de pharynx. Ook de andere twee *ndk* genen, *ndk3* en *ndk4*, komen tot expressie in de pharynx. *Ndk(RNAi)* resulteert in de vorming van ectopische hersenganglia en uitbreiding van de hersenen. In tegenstelling tot *ndk(RNAi)* veroorzaakt de inhibitie van de overige *ndk* genen geen fenotypische afwijkingen. De functies van deze *ndk* genen blijven voorlopig onbekend, maar hun expressiepatroon in de pharynx kan belangrijke informatie bieden over mogelijke rollen van deze genen in de platworm *S. mediterranea*. Fosfohiston H3 immunokleuringen werden uitgevoerd om een mogelijke rol van *ndk* in stamcelproliferatie te onderzoeken. Toenames in het aantal mitoses werden waargenomen in intacte en regenererende *ndk(RNAi)* organismen.

Octanol-blootstellingen werden gebruikt om GJ communicatie in drie planaria soorten te bestuderen. Bipolaire hoofdvorming na kop- en staartamputatie werd echter enkel waargenomen in *D. japonica*. Door soortafhankelijk capaciteiten hadden de octanolbehandelingen geen effect op *S. mediterranea* of *G. tigrina*.

In dit project biedt enkele antwoorden op de vele vragen omtrent regeneratie in planaria. Meer onderzoek is echter nodig om alle informatie te achterhalen omtrent signalering en de betrekkingen van de *nou-darake* genen in platwormregeneratie.

I. Introduction

1. Planarians as model organisms

Due to their therapeutic potential for curing degenerative diseases and repairing injuries, the interest in stem cells increased dramatically over the last few years. However, before human stem cells can be effectively and safely applied in a clinical context, several fundamental questions about the basic biology of stem cells need to be addressed. What in the microenvironment of the stem cell controls its proliferation and differentiation? How are the developmental potentials of stem cells restricted to a particular fate? How is pluripotentiality maintained and what steps lead to its loss? [1]

Planarians have become essential model organisms in the scientific world and these animals have been used to study development and regeneration for more than 200 years [2,3]. A number of established methodologies and recent technical advances make planarians an excellent model system to address the above questions.

1.1. Phylogeny and morphology

Planarians are free-living representatives of the phylum Platyhelminthes, a group of some 50,000 species of flatworms. Flatworms are among the simplest bilaterally symmetric animals. These organisms are acoelomates, yet they have derivatives of all three germ layers organized into complex organ systems. Current models place the Platyhelminthes in a large assemblage of protostome invertebrates, known as the Lophotrochozoa, a sister group to the Ecdysozoa. *Schmidtea mediterranea, Dugesia japonica* and *Girardia tigrina* **(figure 1)** are all freshwater species belonging to the Dugesiidae family. Planarians are unsegmented and dorsoventrally flattened soft-bodied organisms, without circulatory, respiratory, or skeletal structures [2-4]. The planarian central nervous system (CNS) is composed of a two-lobed brain or cephalic ganglia and a pair of longitudinal ventral nerve cords (VNCs). The brain has an inverted U-shaped structure with multiple branches on each outer side. Two eyes are located on the dorsal side at the level of the third branch. The sixth to ninth branches of each brain side cluster more closely and form auricles on the surface, which may function as a sensory organ of taste. The VNCs, which are connected to each other on the ventral side of the brain, run along the length of the animal. Along the VNCs,

small clusters of neurons are grouped in more or less regularly spaced ganglia. From these ganglia, transverse commissures extend connecting both VNCs. Also lateral nerves extend from the VNC and form bundles that branch near the edge of the animal [5] **(figure 2-A).** The gastrointestinal tract of *S. mediterranea* consists of a mouth located at the ventral side of the pharynx, a prepharangeal gut branch and two postpharangeal gut branches **(figure 2-B).**

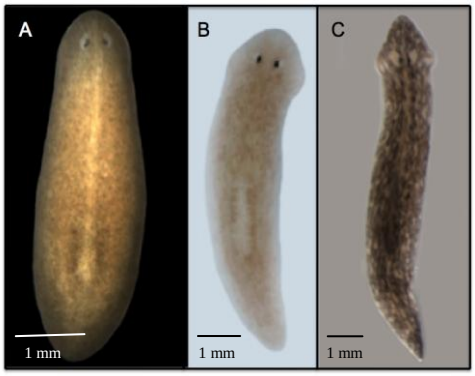


Figure 1: A) *Schmidtea mediterranea*, B) *Dugesia japonica*, C) *Girardia tigrina.*

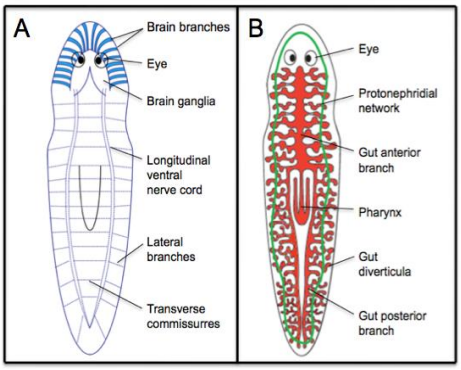


Figure 2: A) Planarian nervous system, B) Planarian gastrointestinal stystem.

1.2. Neoblasts

Planarians possess the ability to replace large missing body parts, a process called regeneration. The source of the plasticity and regenerative ability is a dynamic population of adult, pluripotent stem cells known as neoblasts. Neoblasts are small, highly undifferentiated cells with large nuclei and very little cytoplasm. These stem cells represent 25-30% of all cells in the body of the flatworm. Neoblasts are the only mitotically active cells in adult planarians and they give rise to all the cell types found in the organism, including the germ line. Somatic tissues are constantly being replaced by stem cell proliferation and differentiation [6-8]. *Schmidtea mediterranea, Dugesia japonica and Girardia tigrina* have experimentally accessible populations of stem cells, which make these animals ideal model organisms to access and study these cells *in vivo*. Without interrupting the microenvironment in which stem cells reside, critical regulatory signals for their proliferation and differentiation can be investigated. The accessible stem cell population, coupled with RNA interference (RNAi) injecting double-stranded RNA (dsRNA) through microinjections, will provide a surrogate system and an excellent model for analyzing the basic mechanisms that control stem cell behavior.

2. Anterior/posterior polarity

When amputated along any plane, planarians are capable of regenerating all missing tissue to restore the original anterior-posterior (AP) polarity. These organisms even possess the ability to regenerate an entire brain that is functionally integrated with the rest of the body in just five days [9]. Recently, rapid progress has been made in understanding the developmental pathways involved in planarian regeneration.

2.1. FGFR1/FGFR2

The fibroblast growth factors (FGFs) are small monomeric proteins that regulate a variety of cellular functions, including proliferation, differentiation, cell migration and apoptosis. FGF signalling is therefore involved in multiple biological processes such as embryonic development, organogenesis, angiogenesis, wound healing and tumor formation. The FGFs bind to different FGF receptors (FGFRs) belonging to the receptor tyrosine kinase family. Two FGFR family members have been identified in planarians: FGFR1 and FGFR2. The FGFRs possess three extracellular immunoglobuline (Ig)-like loops, a single transmembrane domain and an intracellular tyrosine kinase domain. In intact worms, expression of both genes has been observed in the brain and in the mesenchymal space [10]. The *FGFR1* gene is expressed in the blastema[1] and regenerating brain and pharynx tissues in regenerating planarians. These data indicate that FGFR1-positive cells may play roles in both blastema formation and regenerating organs. Furthermore, irradiation experiments showed that FGFR1 is not only expressed in neoblasts, but also in differentiated (brain) tissue. Expression of FGFR2 during regeneration is observed in the regenerating brain, but not in the blastema or regenerating pharynx. Knocking down the expression of FGFR1, FGFR2 or even both genes does not cause abnormalities in the regeneration process. All dsRNA-injected animals regenerated a normal blastema and pharynx and when compared with control animals [10].

2.2. Ndk

The planarian gene *nou-darake* (*ndk*, Japanese for "brains everywhere) encodes a FGFR-like (FGFRL) protein. Similar to the FGFRs, NDK is a transmembrane protein possessing three extracellular Ig-like domains **(figure 3-A)**. However, it does not own

[1] A mass of undifferentiated cells formed after amputation, capable of regenerating the missing tissue [7].

an intracellular protein tyrosine kinase domain, but instead harbors a C-terminal domain of only 100 amino acids in length that cannot signal by transautophosphorylation [9,11-13]. This protein is specifically expressed in the head region, in both brain and non-brain tissues. Loss of function of the *ndk* gene by RNA interferences results in the formation of ectopic brains throughout the body of regenerating worms. Differentiation of ectopic brain tissues and extra eyes are also observed in intact, non-regenerating animals after injection of *ndk* dsRNA. In double *ndk/FGFR1* and *ndk/FGFR2* dsRNA-injected organisms, ectopic brain tissues are detected, although at a level slightly lower when compared to *ndk* dsRNA-injected animals. In triple-injected worms, *ndk/FGFR1/FGFR2(RNAi)*, no ectopic brains are formed in posterior body regions. These results suggest that NDK may have the ability to bind to FGF or FGF-like molecule(s) and modulate FGF signalling. Based on these observations a speculative model, named the 'capture model' was made **(figure 3-B)**. NDK is specifically expressed in the head region and may regulate the diffusion range of brain activators (FGF or FGF-like molecules) from a putative source in the head region to the rest of the body through direct interaction. Loss of function of *ndk* would allow these factors to travel to more posterior regions, and thus activate FGF receptors outside the head region to trigger ectopic brain formation [9].

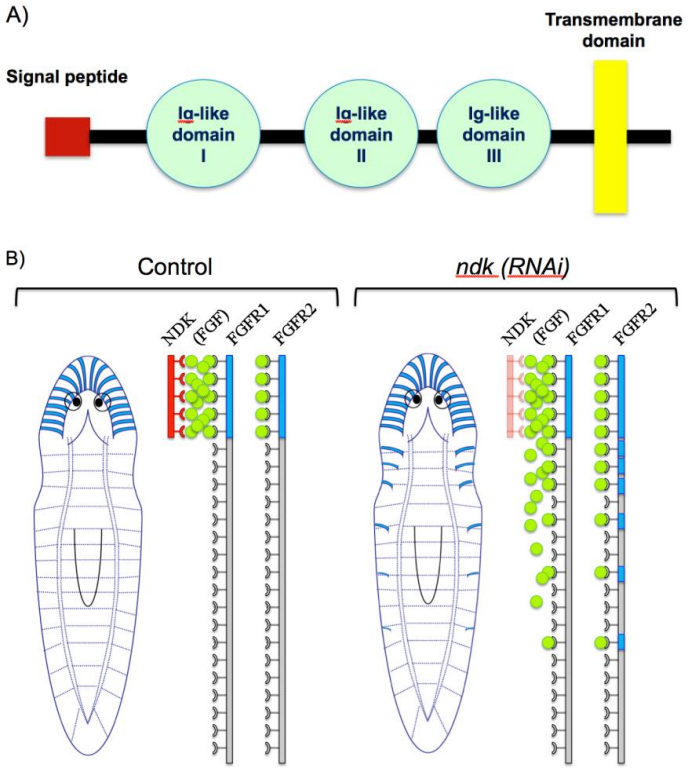


Figure 3: A) Schematic drawing of the Nou-darake (NDK) protein. NDK has a signal peptide, three extracellular immunoglobin (Ig)-like domains, a transmembrane domain and a short intracellular domain.
B) The capture model: the figure on the left shows a control animal in which NDK is specifically expressed in the head region and may regulate the diffusion range of brain activators (fibroblast growth factors, FGF). In the right animal, *ndk* is knocked down, allowing the factors to travel to posterior regions. This activates FGF receptors outside the head region, triggering ectopic brain formation (Agata & Umesono, 2008) [9].

2.3. Wnt/ β-catenin pathway

Wnt/β-catenin signalling is central in promoting posterior specification and inhibiting anterior identity in planarians. Wnt proteins are a family of highly conserved secreted glycoproteins that control cell-to-cell interactions during embryogenesis and regeneration. Canonical Wnt signalling is tranduced through β-catenin, a multi-functional protein regulating transcriptional outputs. In response to upstream signalling initiated by Wnt ligands binding to frizzled receptors, β-catenin will induce transcriptional responses that direct specification and patterning. APC is an essential member of the destruction complex that phosphorylates β-catenin, resulting in its degradation. The binding of Wnt ligands to the frizzled receptors prevents the formation of the destruction complex and therefore no phosphorylation of β-catenin can occur [14-22]. Via this Wnt/β-catenin pathway, Hedgehog (Hh) signalling plays a crucial role in posteriorization by regulating the transcription of the posterior-specific *wnt* genes. Secreted Hh protein binds to the cell-surface receptor patched (Ptc), preventing repression of the seven-membrane spanning receptor smoothened (Smo). This will lead to the activation of Gliotactin (Gli) transcription factors, promoting the transcription of posterior-specific *wnt* genes [23,24] **(figure 4).**

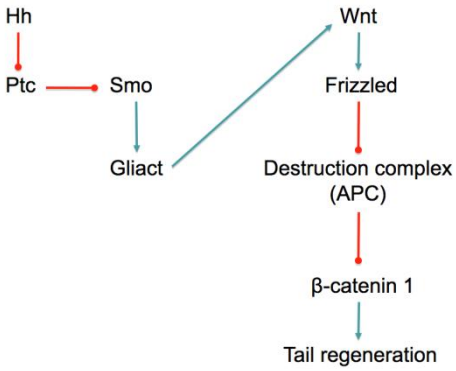


Figure 4: Pathways regulating tail regeneration; The hedgehog (Hh) gene regulates the transcription of posterior-specific Wnt genes by activating Gli transcription factors. These Wnt proteins bind to frizzled receptors, inhibiting the formation of the destruction complex. This prevents the phosphorylation of β-catenin, and transcription of the posterior-specific genes can occur.

Wnt signalling acts in a gradient over the anterior/posterior axis to specify the correct fates during regeneration and homeostasis [25]. Due to the presence of an active β-catenin gradient, knock down of *β-catenin* results in loss of this gradient, creating animals with two heads of opposite orientation [19,20]. Since *apc* knock down prevents the formation of a functional destruction complex, *apc(RNAi)* animals regenerate tails from both the anterior and posterior amputation plane. In *hh* dsRNA-injected worms, repression of Smo does not occur, preventing the transcription of the *wnt* genes. Amputation of the head and tail after *hh(RNAi)* results in a tailless phenotype. Loss of Wnt activity leads to ectopic formation of anterior structures at all wounds and gradual homeostatic anteriorisation of the whole axis [22,24] **(appendix A).**

2.3.1. Innexins

Planarian gap junction (GJ) proteins, or innexins, are plasmamembrane channels involved in direct cell-cell communication. GJ protein-mediated signals regulate behavior of embryonic and adult stem cells by modulating the exchange of information between undifferentiated cells and their surrounding microenvironment. Such short-range stem cell regulation by GJ proteins is essential for regeneration of complex structures in both vertebrates and invertebrates, but it is also possible that these GJ proteins play important roles in long range signalling [26-28]. The functional role of GJ communication during planarian regeneration was first described by Nogi T. & Levin M. (2005). They show that worms exposed to heptanol (7-OH) during the first 2 days of regeneration exhibited significant anteriorization, ranging from an inhibition of tail development to the appearance of a complete second head at the posterior blastema. These data suggested that GJ are involved in determination of anterior-posterior identity during regeneration [26]. Oviedo et al. (2010) established that both the central nerve system and innexins play critical roles in the long range signalling during regeneration. By exposing animals of *Dugesia japonica* to an octanol solution (8-OH, a GJ blocker), the importance of this long range signalling pathway could be examined. They noticed that in the case of regenerating head-pieces, untreated control animals, octanol-exposed animals and animals with VNC disruption regenerated the missing posterior area with normal polarity. However, in animals in which both octanol treatment and VNC disruption took place, signals coming from anterior areas were disrupted, leading to abnormalities in polarity (bipolar animals). When examining post-pharyngeal fragments with anterior and posterior-facing wounds, animals exposed to octanol regenerated bipolar heads. In the case of VNC disruption alone, most worms regenerated without abnormalities. The data suggest that these long range pathways are signalling the presence or nascent formation of a head elsewhere in the planarian. These results are consistent with the default identity for wounded surfaces being “head formation”. Information instructing the neoblasts that a head is already present elsewhere reaches the blastema via two routes: through the VNCs, and innexin proteins [28].

3. Brain regeneration

The process of brain regeneration in planarians can be divided in 5 steps. Firstly, after decapitation the anterior blastema forms following wound closure. Secondly, a brain rudiment is formed in this blastema. These first two steps occur within 24 hours after amputation. After 36-48 hours, the brain rudiment undergoes pattern formation. In step 4, the brain and the VNCs, and the brain and the eyes become connected to each other 72 h after the decapitation. The network structure of the CNS is completely reformed within 4 days. Although morphological renewal is completed within 4 days, an additional day is needed for functional recovery [9,29-31].

It is observed that in animals regenerating two tails after *apc(RNAi)* or *ptc(RNAi)*, early ectopic brain primordia occur during regeneration [32]. To distinguish the source of the cells forming these early brains observed in *apc(RNAi)* and *ptc(RNAi)* animals, double cutting experiments were performed. The observation that double cuts stop the formation of early brains revealed that these brain primordial may be formed from uncommitted adult stem cells at later phases of the cell cycle at the time of amputation. To test this possibility, hydroxyurea (HU) treatments were performed combined with RNAi experiments. HU blocks the transition of neoblasts through the cell cycle at S-phase. Control, *apc(RNAi)* and *ptc(RNAi)* HU treated animals were still able to regenerate brain primordia, however they all failed to elaborate these structures. Colchicine treatment, which stops cells transiting through G2/M, resulted in animals unable to produce brain structures. This suggests that early Wnt independent brain primordia form from adult stem cells that are in the G2/M phase of the cell cycle at the time of decapitation. These cells are able to read their position in remaining tissue through an as yet unidentified mechanism and those in the most anterior post-blastema region begin to differentiate into brain primordia, independently of Wnt-specified regenerative polarity. This is confirmed by experiments combining *β-catenin(RNAi)* and HU exposure. *β-catenin(RNAi)* animals treated with HU did form early anterior brains but not the characteristic ectopic brain structures, while control *β-catenin(RNAi)* animals produced brains in both blastemas. These data show that ectopic anterior structures in the posterior resulting from disruption of Wnt activity form from a population of stem cells at a different stage of the cell cycle to those able to form early anterior brain.

4. Objectives

Since planarians possess the ability to regenerate a functional bilateral brain within five days after decapitation, they are perfect model organisms to investigate the complex process of brain regeneration. Planarians are simple organisms, however, studying their regenerative capacities should provide important insights into the fundamentals of brain regeneration across species. Although much progress has been made over the last years, much remains to be discovered. In this study we investigated the importance of both short and long range signalling in brain regeneration.

Four *ndk* genes have been discovered in *S. mediterranea*, of which three have been described in the literature: *ndk*, *ndk3* and *ndk4*. The forth gene, which we named *ndk2*, was unknown in scientific literature. In this research, we explored the expression pattern of this NDK2 and compared it with the other three NDKs. Is the expression pattern of NDK2 similar to that of NDK1, or does it resemble the expression pattern of one of the remaining NDKs?

In previous experiments performed in cooperation with Aziz Aboobaker, it was shown that *ndk(RNAi)* resulted in the formation of two early ectopic brain ganglia in the posterior blastema after 72 hours of regeneration. RNAi experiments combined with HU treatments will be performed to study the role of these genes on early ectopic brain regeneration. Do *ndk(RNAi)* animals exposed to HU still form the ectopic brains? Are *ndk2(RNAi)*, *ndk3(RNAi)* and *ndk4(RNAi)* worms also able to produce these ectopic brain ganglia? How do these organisms react to the HU treatments?

Furthermore, we will investigate the effect of the RNAi treatments later on in the regeneration process. Do *ndk2(RNAi)*, *ndk3(RNAi)* or *ndk4(RNAi)* cause a similar brain expansion seen in *ndk(RNAi)* animals after 14 days of regeneration?

Last, we will explore the role of long range signalling in various planarian species, including both sexual and asexual strains of *Schmidtea mediterranea*, the sexual strain of *Dugesia japonica* and the sexual strain of *Girardia tigrina*. Oviedo et al. (2010) showed that innexins play a crucial role remaining the correct polarity during regeneration in *D. japonica*. Can the same results be obtained in both strains of *S. mediterranea* and in *G. tigrina*?

II. Materials and methods

1. Materials and solutions

All the materials used in this research project are alphabetically listed in appendix A. Both the company as the product number can be found in this table. Appendix B lists all the solutions and buffers and their compositions used in the different protocols.

2. Animals

Three different species of planarians were used in this research project: *Schmidtea mediterranea, Dugesia japonica* and *Girardia tigrina.* The animals of the *S. mediterranea* and *G. tigrina* species were provided to the Aboobaker lab by Professor Dr. E. Salo. The *D. japonica* worms were given by Professor Dr. T. Hayashi. All animal cultures were maintained at a constant temperature of 20º C in tap water treated with activated charcoal and buffered with 0.5 mM $NaHCO_3$. The animals were fed weekly with veal liver and starved for at least one week prior to the experiments.

3. Experimental set-up

3.1. Microinjections

Depending on the experiment, one round or two rounds of injections were performed. In each round, every animal was injected with 3 x 32.2 nl of double-stranded RNA (dsRNA) for three consecutive days **(figure 5)**. Amputations were performed one day after the last day of injections. The dsRNA was injected at a concentration of 2 μg/μl. For the double knock-out experiments, both genes were injected at this concentration, resulting in a total dsRNA concentration of 4 μg/μl. The concentrations of the controls in the double knock-out experiment were equally adjusted. Injections were performed using a Drummond Scientific Nanoject injector [33 & 34].

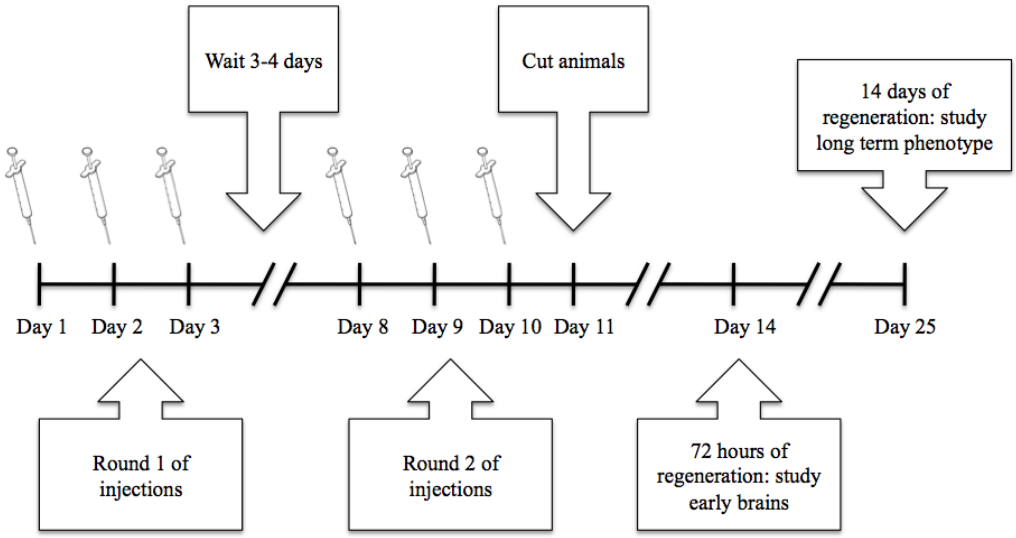


Figure 5: Injection schedule. After the first round of injections, the animals were left alone for four days before injecting them for another three days. One day after the second round of injections, amputations were performed and animals could regenerate and be fixed.

3.2. Hydroxyurea treatment

The effect of hydroxyurea (HU) on early posterior brain development was investigated by exposing the animals for 16 hours to a HU concentration of 20 mM 6 hours after the last injections.

3.3. Octanol treatment

The effects of long range signalling were studied by exposing three different planarian species to octanol. Animals were exposed to the indicated concentrations of octanol for 3 days immediately after cutting. After the octanol exposure, asexual *S. mediterranea* worms were regenerated for 14 days and *D. japonica, G. tigrina* and sexual *S. mediterranea* worms were regenerated for 21 days in planarian water [28].

4. dsRNA and in situ *probe preparation*

4.1. RNA extraction and cDNA preparation

Worms at different regeneration time points were incubated overnight at -20º C in 300 µl Trizol. The animals were ground the next day, after which 700 µl Trizol was added. The solution was incubated for 10 minutes at room temperature (RT) before 200 µl chloroform was added. The sample was then incubated for 5 minutes at RT and centrifuged (15 minutes, 13,000 rpm, 4º C). The upper aqueous phase was

collected and combined with 500 μl of isopropanol. The mixture was incubated at -20º C and centrifuged before the pellet was washed with cold 75% ethanol. To synthesize the cDNA, the pellet was air-dried and resuspended in 20 μl ultraPURE H_2O. Reverse transcription was performed in a 20 μl volume with 2.5 mM oligo dT primer, 1 μg of total RNA, 0.5 mM dNTP. The solution was incubated for 5 minutes at 65º C after which 4 μl of 5 x 1st strand buffer, 5 mM DTT, 1 μl RNase inhibitor and 1 μl Superscript III was added. The reaction was incubated at 50º C for 2 hours. The reaction was inactivated at 70º C for 15 minutes. Finally, 1 μl Turbo DNase was added and the solution was incubated at 37° C for 30 minutes. The reaction was heat inactivated at 75° C for 10 minutes.

4.2. Identification of the ndk *genes in* S. mediterranea *and primer design*

The sequences of the *ndk, ndk3* and *ndk4* genes from *S. mediterranea* were downloaded from the NCBI database. The forth *ndk* gene named *ndk2* was identified by querying the *S. mediterranea* transcriptome database [35] with the *D. japonica ndk* sequence using the tblastn algorithm. The primers sequences of all the genes used in this research project for the preparation of both dsRNA as RNA probes, are listed in appendix C.

4.3. Cloning from cDNA

A 20 μl PCR reaction was performed containing 2 μl 10 x Buffer, 0.2 mM dNTPs, 1 μl Taq Polymerase, 200 ng cDNA and 1 mM of both the forward and reverse primer flanking the sequence of interest. A second PCR reaction (50 μl) was performed on a 1/10 dilution of the product of this reaction. PCR products were purified using a QIAquick gel extraction kit. Ligations were performed using the Quick Ligation kit. Competent *E. coli* cells were transformed by adding 1 μl of ligation to 40 μl of cells and incubate for 30 minutes on ice, after which the cells were heat shocked at 42º C for 1 minute. The cells were chilled on ice for 5 minutes, after which 500 μl of media was added. The transformed cells were incubated at 37º C for 1 hour. Subsequently, 200 μl of the culture was spread out onto IPTG-X-gal and ampicilin containing agar plates. These plates were incubated overnight at 37º C. The colonies containing the PCR fragment ligated into the vector were identified by their white colour. Positive colonies were grown overnight at 37º C in liquid medium containing ampicillin. The plasmid was obtained by using the QIAprep spin miniprep kit. To synthesise linear

DNA from the plasmid templates, a 20 µl PCR reaction containing 0.2 mM dNTPs, 2 µl M13 forward primer, 2µl M13 reverse primer, 2 µl 10 x Buffer, 0.2 µl Taq Polymerase, 1µl of purified DNA was performed. The obtained PCR products were purified using the QIAquick gel extraction kit.

4.4. RNA probe preparation

To synthesise RNA probes, transcription was performed in a reaction containing 1 µl 10 x Transcription Buffer, 1 µl 10 x DIG labelling mix, 1 µl T7 or Sp6 RNA polymerase, 0.25 µl RNase Inhibitor and 300 ng of purified PCR product in a reaction volume of 10 µl. The reaction was incubated for 2 hours at 37º C. Next, 1 µl of Turbo DNase was added and the reaction was incubated for a further 30 minutes at 37º C. RNA was precipitated from solution by adding 30 µl ultraPURE H_2O, 5 µl LiCl (4M), 150 µl cold 100% ethanol and 1 µl glycogen and incubating at -20º C for 2 hours. The sample was centrifuged (20 minutes, 13,000 rpm, 4º C) and the pellet was washed using cold 100% ethanol. The pellet was air-dried and resuspended in 20 µl resuspension buffer by incubating at 65º C for 10 minutes.

4.5. dsRNA preparation

Double-stranded RNA was synthesised in a reaction containing 2 µl 10 x Transcription Buffer, 2 µl 10 x rNTPs, 2 µl T7 or Sp6 RNA polymerase, 1 µl RNase inhibitor and 500-1000 ng of the purified PCR product in a total volume of 20 µl which was incubated at 37º C for 4 hours. Next, 1 µl of Turbo DNase was added and the solution was again incubated at 37º C for 30 minutes. The products of the T7 and Sp6 polymerase reactions were combined and and precipitated together by adding 6 µl sodium acetate (3M) and 120 µl cold 100% ethanol and incubating at -80º C for 2 hours. The precipitated RNA was pelleted by centrifugation (15 minutes, 13,000 rpm, 4º C). The pellet washed with cold 70% ethanol, air-dried and resuspended in 10 µl ultraPURE H_2O. The dsRNA was incubated at 68º C for 15 minutes followed by 45 minutes at 37º C.

5. Whole mount in situ *hybridization protocol*

Two fixation processes were applied during this research project. For fixation with Carnoy's solution, mucus was removed in ice cold 2% HCl / ⅝ Holtfreter for 5 minutes before fixation in Carnoy's solution for 2 hours at 4º C. For fixation with

formaldehyde, mucus was removed in 5% N-acetyl cysteine (NAC)/PBS solution for 5 minutes at RT before fixation in 4% formaldehyde/PBST (PBS, 0.1% TritonX-100) for 20 minutes at RT. Animals were rinsed with PBST before being placed into preheated reduction solution for 10 minutes at 37° C. The samples were gently agitated by hand during the reduction. Animals were then rinsed with PBST and the PBST was replaced by 50% methanol/PBST for 10 minutes at RT [36]. After fixation by either method the animals were transferred into 100% methanol for 1 hour at -20º C. To remove the pigmentation, the worms were bleached in 5% H_2O_2/methanol by illumination.

Animals were rehydrated through a series of ethanol washes: 70% ethanol/ ⅝Holtfreter, 50% ethanol/ ⅝Holtfreter and 30% ethanol/ ⅝Holtfreter respectively, each for 30 minutes at 4º C. Animals were transferred into TPBS and washed for 20 minutes at 4º C after which they were exposed to 20 μg/ml proteinase K/TPBS for 5 minutes at 37º C. The proteinase K/PBST was removed with two ⅝Holtfreter washes and the animals were exposed to 4% paraformaldehyde (PFA)/ ⅝Holtfreter for 1 hour at 4º C. Animals were then washed in ⅝Holtfreter for 1 hour at 4º C. Tissues were acetylated by incubation in 0.1 M TEA (2 x 15 minutes, RT) after which 25 μl of acetic anhydride was added (2 x 15 minutes, RT). The animals were washed with PBS (2 x 10 minutes, RT) before being incubated in prehybridization buffer for 1 hour at 56º C in 1.5 ml epindorphs. Hybridization was carried out for 16 hours at 56° C in hybridization buffer. All *in situ* probes were used at a concentration of 500 ng/μl.

Samples were then washed through a series of posthybridization buffers: PostHyb1, PostHyb 2, PostHyb 3 and PostHyb 4, each for 10 minutes at 56º C. Samples were then washed for 2 x 30 minutes in PostHyb5 and 2 x 30 minutes in PostHyb6 at 56º C. The worms were washed for 2 x 10 minutes at RT in Buffer I and blocked in Buffer II for 1 hour at RT in a 24 well plate. Samples were then incubated at RT for 3 hours in 1:2000 antiDIG/Buffer II. The antibody was removed by washing with Buffer I for 2 x 10 minutes at RT and overnight at 4° C. The worms were washed with Buffer I for a further 2 x 10 minutes at RT after which they were incubated in TMN for 5 minutes at RT. Colour development was performed by incubation of the samples in 20 μl/ml NBT/BCIP at RT. When the colour reaction was complete, the animals were washed with PBS for 2 x 10 minutes at RT and then fixed in 4% PFA/PBS for 30 minutes at

RT. Next, a series of ethanol washes of 30% EtOH/PBS, 50% EtOH/PBS, 70% EtOH/PBS, 100% EtOH/PBS, 70% EtOH/PBS, 50% EtOH/PBS and 30% EtOH/PBS was performed, each for 5 minutes at RT. Finally, the worms were washed in PBS for 2 x 5 minuts at RT, mounted onto glass slides in 70% glycerol/PBS and imaged on a Zeiss Discovery V8 from CarlZeiss using Axio Cam MRC from Carl Zeiss.

6. Immunohistochemistry

Samples were fixed in Carnoy's fixative as described for whole mount *in situ* hybridization. Animals were washed with 75 %, 50 % and 25 % methanol/PBST solutions, each for 10 minutes at RT. Samples were washed for 2 x 10 minutes at RT in PBST and the animals were blocked in 1 % BSA/PBST for 2 hours at RT. Samples were incubated in primary antibody (1:1000 dilution of Rabbit anti-phospho histone H3 mAb) for 48 hours at 4º C. The primary antibody was washed off with 7 PBST washes, each for 30 minutes at RT. The worms were blocked in 1% BSA/BPST for 1 hour at RT followed by incubation with the secondary antibody (1:1000 dilution of Alexafluor goat anti-rabbit) for 14 hours at 4º C. Following 6 x 30 minutes PBST washes at RT, the worms were mounted in 70% glycerol. Fluorescent images were taken on a Leica MZ16F fluorescence stereomicroscope with a Leica DFC 300Fx camera.

7. Statistics

One-way ANOVA tests were carried out to determine the statistical significance of the effect of *ndk(RNAi)* on stem cell proliferation.

The *nou-darake (ndk)* genes in the planarian *Schmidtea mediterranea*

III. Results

1. *The expression patterns of the* ndk *genes*

1.1. Expression of the ndk *genes in intact planarians*

Whole mount *in situ* hybridizations were performed to obtain the expression patterns of the *ndk* genes in intact animals of the planarian species *Schmidtea mediterranea*. Worms were fixed using Carnoy and formaldehyde fixation methods (see materials and methods). The expression patterns are presented for both fixation methods in **figure 6**.

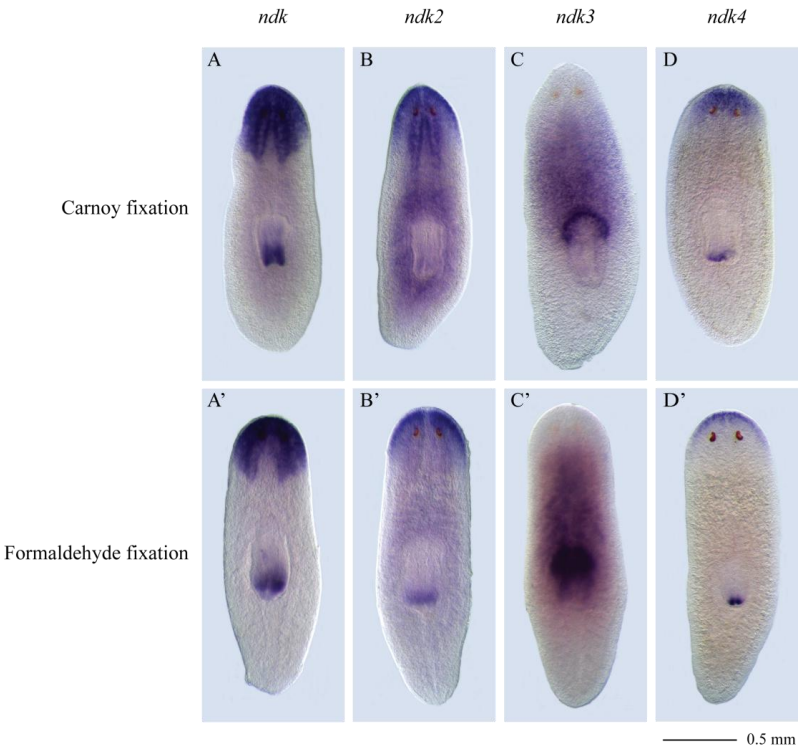


Figure 6: *Ndk, ndk2, ndk3* and *ndk4* expression patterns in intact organisms of *S. mediterranea* using Carnoy and formaldehyde fixation methods. A-D: Carnoy-based whole mount in situ hybridization method; A'-D': Formaldehyde-based whole mount *in situ* hybridization method. A & A': *Ndk* expression patterns; both fixation processes show expression of *ndk* in brain tissue and in the pharynx. B & B': *Ndk2* expression patterns; the Carnoy-based fixation shows staining in the head region in the brain; expression in the VNCs and the area surrounding the pharynx is also observed. A staining surrounding the pharynx is detected using both fixation methods, but is less obvious in formaldehyde-fixed animals. C & C': *Ndk3* expression patterns; both fixation processes show expression of *ndk3* in the pre-pharyngeal region. Expression of the gene is also observed in the pharynx, but in contrast with the other *ndk* genes it is expressed in the anterior part of the organ. D & D': *Ndk4* expression patterns; both fixation patterns show similar *ndk4* expression patterns in the area above the photoreceptors and in the pharynx. Scale bar: 0.5 mm.

Figure 6 A and A' show the expression patterns of *ndk* for both fixation processes. These figures demonstrate that *ndk* is expressed in the head, in agreement with the data of Francesc Cebrià (2002) [12]. Both the Carnoy- and formaldehyde-based fixation process show colour development in the pharynx, indicating that *ndk* is not only expressed in the head region but also in the pharynx.

The expression patterns of *ndk2* are similar but not completely identical in the two fixation processes. Both methods show *ndk2* expression in the head region. However, the formaldehyde-based fixation reveals expression of this gene in the pharynx, which is not observed in the method using Carnoy's solution as the fixative. The staining surrounding the pharynx, which is detected using the Carnoy-based fixation, is also observed in the formaldehyde-fixed animals, though less obviously **(figure 6, B and B').**

Both fixation processes show *ndk3* expression in the pre-pharyngeal area but not the head region, as well as in the pharynx. However, contrary to the other *ndk* genes, the expression of *ndk3* in the pharynx is located anteriorly in the organ **(figure 6, C and C').**

The expression patterns of *ndk4* are similar for both fixation processes, showing expression of this gene in the head anterior to the photoreceptors. No expression is observed in brain tissue. Comparable to the expression of *ndk* and *ndk2*, *ndk4* is also expressed in the pharynx **(figure 6, D and D').**

1.2. Expression of the ndk *genes in regenerating planarians*

In situ hybridizations on regenerating animals provide more information regarding the roles of these *ndk* genes on the development and the activation of their expression during regeneration. To gather more insight on the expression patterns of all *ndk* genes during regeneration, an experiment in which animals were fixed at different regeneration time points (after 1 day, 2 days, 3 days, 5 days and 7 days of regeneration) was performed **(figures 7, 8, 9 & 10).** All the animals used for the time course expression patterns were fixed using Carnoy's fixative.

Figure 7 shows the expression pattern of *ndk* in regenerating head, trunk and tail pieces at the different time points (1 day, 2, days, 3 days, 5 days and 7 days). *Ndk* expression is detected in the regenerating brain ganglia in the anterior blastemas of the trunk and tail pieces after 2 days of regeneration. In the regenerating pharynges in head and tail pieces, *ndk* expression is observed starting at the fifth day of regeneration. Due to technical difficulties, the expression pattern of *ndk* in the regenerating head piece after 2 days of regeneration was not obtained.

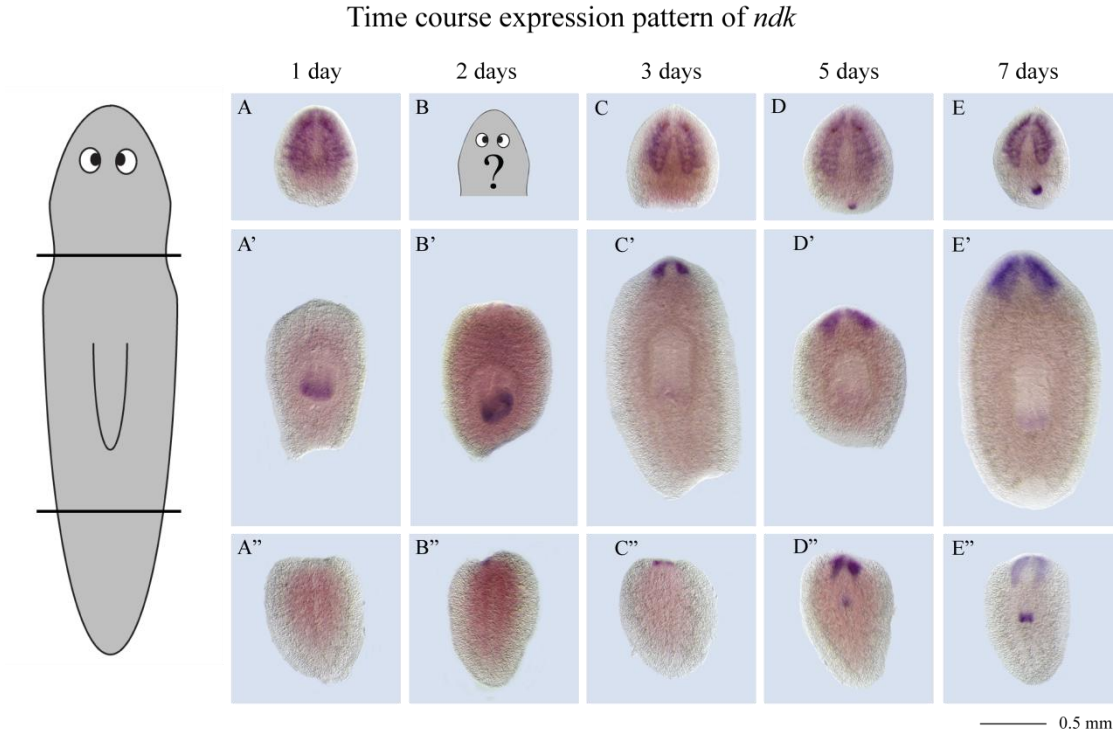


Figure 7: Expression pattern of *ndk* in regenerating animals of *S. mediterranea*. A-A": Expression of *ndk* after 1 day of regeneration; expression of the gene is visible in the pre-existing brain and pharynx. No expression is detected in the blastema. B-B": Expression of *ndk* after 2 days of regeneration. *Ndk* expression is detected in regenerating brain ganglia in the regenerating trunk and tail pieces. Due to technical difficulties, no head pieces after 2 days of regeneration were obtained. C-C": Expression pattern of *ndk* after 3 days of regeneration. Expression of *ndk* is observed in the regenerating brain ganglia of both trunk piece and tail piece. D-D": Expression pattern of *ndk* after 5 days of regeneration. Expression of the *ndk* gene expands together with the regenerating brain. *Ndk* is also expressed in the regenerating pharynges of both head and tail pieces. E-E": Expression pattern of *ndk* after 7 days of regeneration. *Ndk* expression is detected in the regenerating brain ganglia and pharynges. Growth of these regenerating organs correlates with the pattern of *ndk* expression. A-D: Regenerating head pieces. A'-D': Regenerating trunk pieces. A"-D": Regenerating tail pieces. Scale bar: 0.5 mm.

The expression pattern of *ndk2* during regeneration is comparable to *ndk*. Similar to the *ndk* time course expression pattern, *ndk2* expression is observed in both the regenerating brain ganglia and pharynges. After 3 days of regeneration, *ndk2* is expressed in the forming brain ganglia in trunk and tail pieces. At the fifth day of regeneration, expression of *ndk2* is also detected in the regenerating pharynx in both head and tail pieces. In the head pieces, *ndk2* expression is observed in the pre-existing brain while in the regenerating trunk pieces, *ndk2* is expressed in the pre-existing pharynx. The staining surrounding the pharynx is also present in the regenerating trunk pieces, comparable to the *ndk2* expression pattern in intact planarians, **(figure 8)**.

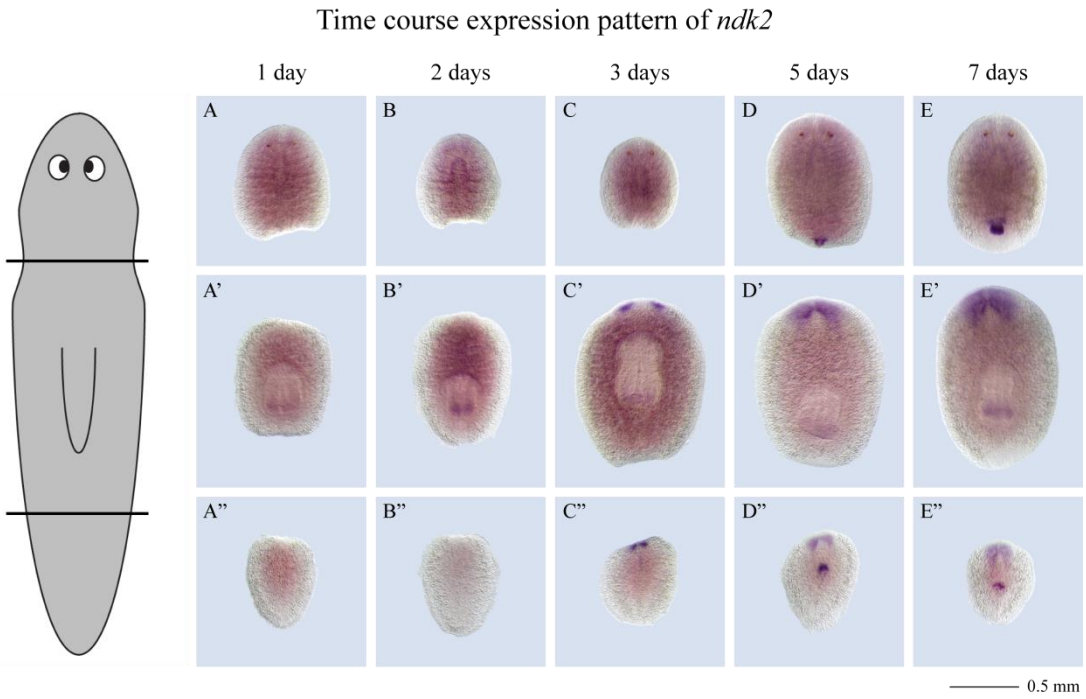


Figure 8: Time course expression pattern of *ndk2* in regenerating animals of *S. mediterranea*. A-A": Expression of *ndk2* after 1 day of regeneration; expression of the gene is visible in the pre-existing brain and pharynx. No expression is detected in the blastema. B-B": Expression of *ndk2* after 2 days of regeneration; no expression is observed in the blastemas, although *ndk2* expression is present in the pre-existing brain and pharynx. C-C": Expression pattern of *ndk2* after 3 days of regeneration. Expression is detected in the regenerating brain ganglia in both trunk pieces and tail pieces. D-D": Expression pattern of *ndk2* after 5 days of regeneration. Expression of the *ndk2* gene expands together with the regenerating brain. *Ndk2* is also expressed in the regenerating pharynges of both head and tail pieces. E-E": Expression pattern of *ndk2* after 7 days of regeneration. *Ndk2* expression is detected in the regenerating brain ganglia and pharynges. Growth of these regenerating organs correlates with the pattern of *ndk2* expression. A-E: Regenerating head pieces. A'-E': Regenerating trunk pieces. A"-E": Regenerating tail pieces. Scale bar: 0.5 mm.

In contrast to *ndk* and *ndk2*, *ndk3* is not expressed in the regenerating brain ganglia. These results meet our expectations, since *ndk3* expression was not detected in the head region in the intact animals. However, expression of *ndk3* is observed in the regenerating pharynges of the head and tail pieces at the fifth and seventh day of regeneration **(figure 9)**. Due to technical difficulties with the *ndk3* probe, the pre-pharyngeal expression pattern of this gene seen in intact planarians is not observed in the regenerating pieces.

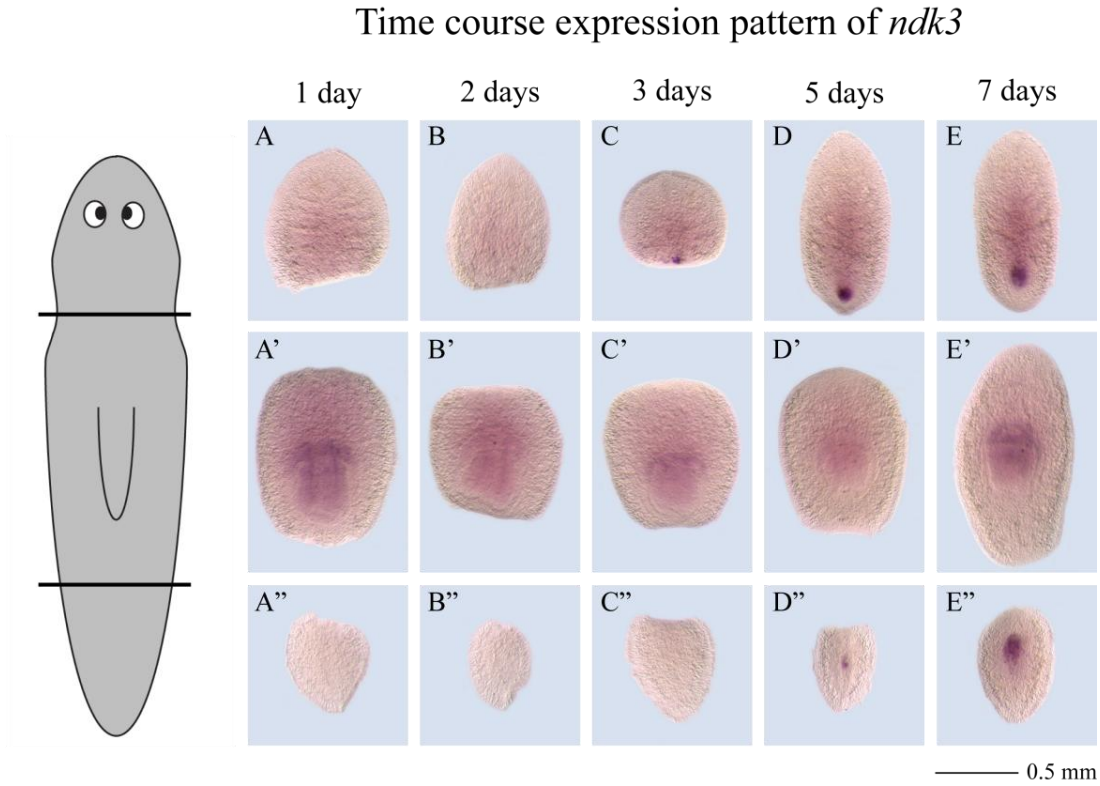


Figure 9: Time course expression pattern of *ndk3* in regenerating animals of *S. mediterranea*. A-A": Expression of *ndk3* after 1 day of regeneration. No *ndk3* expression is detected in either the head, trunk or tail pieces.. B-B": Expression of *ndk3* after 2 days of regeneration; no expression of *ndk3* is observed. C-C": Expression pattern of *ndk3* after 3 days of regeneration. Expression of *ndk3* is detected in the regenerating pharynx in the blastema of the head pieces. D-D": Expression pattern of *ndk3* after 5 days of regeneration. *Ndk3* is expressed in the regenerating pharynges of both head and tail pieces. E-E": Expression pattern of *ndk3* after 7 days of regeneration. *Ndk3* expression is detected in the regenerating pharynges. A-E: Regenerating head pieces. A'-E': Regenerating trunk pieces. A"-E": Regenerating tail pieces. Scale bar: 0.5 mm.

Figure 10 shows the expression pattern of *ndk4* in regenerating *S. mediterranea*. *Ndk4* is expressed in the head region in front of the photoreceptors in regenerating head pieces and in the pre-existing pharynges of the regenerating trunk pieces, comparable with the *ndk4* expression in intact animals. *Nkd4* expression is also detected in the regenerating pharynges of head and tail pieces. Furthermore, *ndk4* is expressed anteriorly in the anterior blastemas of the trunk and tail pieces.

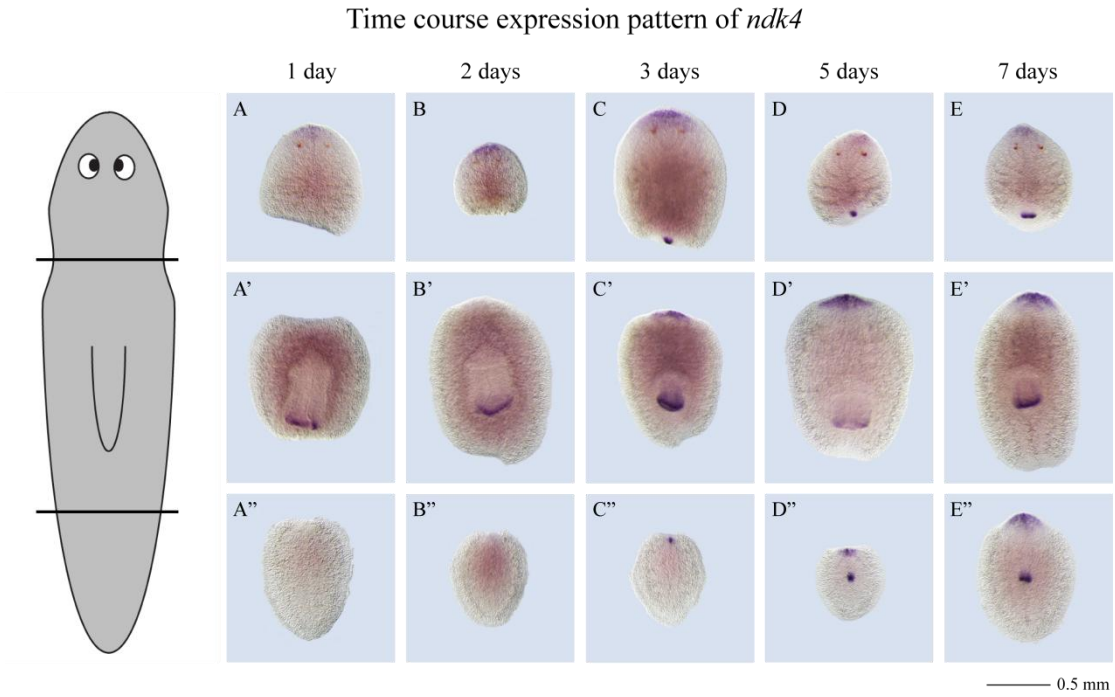


Figure 10: Time course expression pattern of *ndk4* in regenerating animals of *S. mediterranea*. A-A": Expression of *ndk4* after 1 day of regeneration. *Ndk4* expression is visible in the pre-existing brain and pharynx. No expression is detected in the blastema. B-B": Expression of *ndk4* after 2 days of regeneration; no expression is observed in the blastemas, although *ndk4* is expressed in the pre-existing brain and pharynx. C-C": Expression pattern of *ndk4* after 3 days of regeneration. Expression of *ndk4* is detected at the anterior margin of anterior blastemas of both trunk pieces and tail pieces. D-D": Expression pattern of *ndk4* after 5 days of regeneration. Expression of the *ndk4* gene expands as the blastema grows. *Ndk4* is also expressed in the regenerating pharynges of both head and tail pieces. E-E": Expression pattern of *ndk4* after 7 days of regeneration. *Ndk4* expression is detected in the regenerating brain ganglia and pharynges. Expansion of these regenerating organs can be observed with the *ndk4* expression. A-E: Regenerating head pieces. A'-E': Regenerating trunk pieces. A"-E": Regenerating tail pieces. Scale bar: 0.5 mm.

2. Regeneration after ndk *knock down*

To investigate the role of the *ndk* genes in regenerating planarians, RNAi experiments were performed.

2.1. Ndk(RNAi) *induces formation of early ectopic brain ganglia*

Regenerating worms were fixed after 72 hours to investigate the effect of RNAi on early ectopic brain formation. **Figure 12 (A-J)** shows the effect of RNAi for the different *ndk* genes on regenerating head and trunk pieces.

In all the head pieces of *dsRed(RNAi), ndk(RNAi), ndk2(RNAi), ndk3(RNAi)* and *ndk4(RNAi)* regeneration of the pharynx in the posterior blastema is observed (pharynges indicated with arrowheads on **figure 12**). However, only *ndk(RNAi)* results in the formation of two ectopic brain ganglia in this posterior blastema of the regenerating heads (indicated with arrows in **figure 12, B**). Regeneration of the two

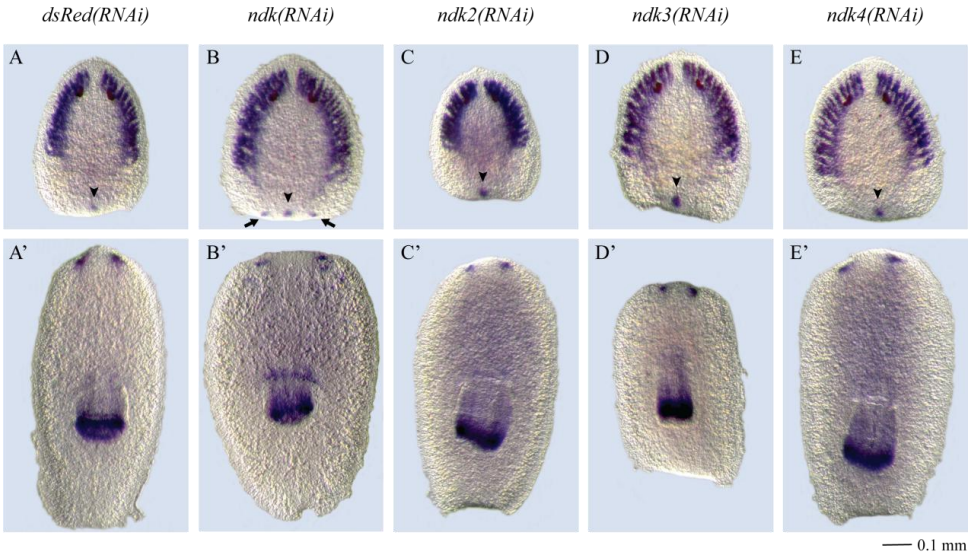
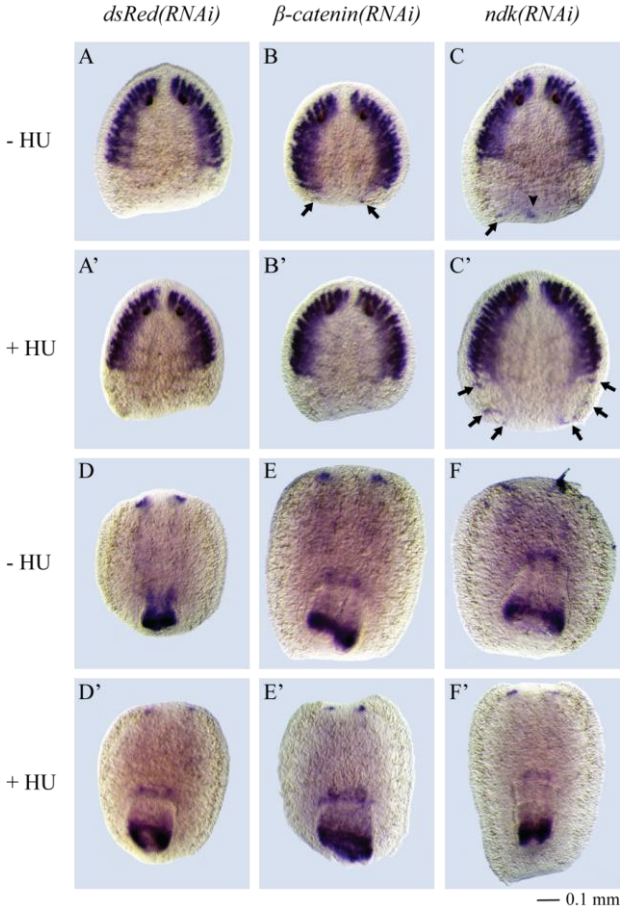


Figure 12: The effect of RNAi of the *ndk* genes on early ectopic brain formation in *S. mediterranea*. A & A': Control animals injected with *dsRed* dsRNA; the regenerating pharynx (arrowhead) is formed in the posterior blatema. Two brain ganglia regenerate in the anterior blastema of the trunk piece. B & B': *ndk(RNAi)* animals; Two ectopic brain ganglia (arrows) form beside a new pharynx (arrowhead) in the posterior blastema of regenerating heads. Two brain ganglia were detected in the anterior blastema of the trunk piece. C & C': *ndk2(RNAi)*; Only the regenerating pharynx (arrowhead) is present in the posterior blastema. Two brain ganglia formed in the anterior blastema of the trunk pieces. D & D': *ndk3(RNAi);* Both head and trunk pieces resembled the control animals with regeneration of the pharynx (arrowhead) in the posterior blastema of the head piece and formation of two brain ganglia in the anterior blastema of the trunk piece. E & E': *ndk4(RNAi)*; Animals are similar to the control pieces, heads regenerating a pharynx (arrowhead) in the posterior blastema, while the trunk pieces formed two brain ganglia in the anterior blastema. A-E: Head pieces; A'-E': Trunk pieces. Scale bar: 0.1 mm.

brain ganglia is detected in the anterior blastemas of the trunk pieces (**figure 12: A'-E'**). Although ectopic brain formation is seen in regenerating head pieces after *ndk(RNAi),* no ectopic brain ganglia are observed in the posterior blastema of the regenerating *ndk(RNAi)* trunk pieces.

To gather more information about the early ectopic brain ganglia resulting from *ndk*(*RNAi),* hydroxyurea (HU) treatments were performed. We used hydoxyurea to block the cell cycle at the G1/S transition to determine whether *ndk(RNAi)* induced posterior ectopic early brains formed specifically from G2/M neoblasts. After one round of injections, animals were exposed to HU for 16 hours before the worms are cut and left to regenerate for 48 hours.



Early ectopic brain ganglia were detected in the posterior blastemas of *β-catenin(RNAi)* and *ndk(RNAi)* head pieces after 48 hours of regeneration in planarian water (indicated by arrows in **figure 13, B & C**). After exposure to a HU solution, no ectopic brains are detected in the *β-catenin(RNAi)* worms at this time point during regeneration. Small ectopic brain ganglia were visible at this regeneration time point in the *ndk(RNAi)* worms (indicated with arrows in **figure 13, F**). Although ectopic brain ganglia in the posterior blastema of the head

Figure 13: The effect of HU exposure on early ectopic brain formation after 48 hours of regeneration in *S. mediterranea.* A, A', D & D': Control animals injected with *dsRed* dsRNA. B, B', E & E': *β-catenin(RNAi)* animals. In the regenerating head piece that was not exposed to HU, ectopic brain ganglia can be detected in the posterior blastema (indicated with arrows in picture B); the regenerating trunk pieces resemble those of the *dsRed(RNAi)* animals with formation of the brain ganglia in the anterior blastemas. C, C', F, & F': *ndk(RNAi)* animals. Comparable with the *β-catenin(RNAi)* animals, ectopic brains (indicated by arrows in picture C) are formed in the posterior blastema of the regenerating head pieces without exposure to HU; in the regenerating head pieces which have been exposed to HU, brain expansion is observed (indicated with arrows in picture C'). A-C': Regenerating head pieces; A-C: No HU exposure; A'-C': HU exposure. D-F': Regenerating trunk pieces; D-F: No HU exposure; D'-F': HU exposure. Scale bar: 0.1 mm.

pieces were observed, significant brain expansion was also detected at this time point 6 days after the initial *ndk* dsRNA injection (3 days of injections, 16 hours HU exposure before cutting and 2 days of regeneration). Early ectopic brain ganglia were not detected in the posterior blastemas of the regenerating trunk pieces of neither *β-catenin(RNAi)* nor *ndk(RNAi)* animals.

2.2. Ndk(RNAi) *animals form posterior brain expansions*

Injected worms that regenerated for 14 days provide more information about the effects of the RNAi treatments. Worms that regenerated for 14 days after *dsRed(RNAi), ndk(RNAi), ndk2(RNAi), ndk3(RNAi)* or *ndk4(RNAi)* treatments were used to study the effects of these genes on complete brain regeneration. **Figure 14 (A-O)** illustrates the structure of the brain in these dsRNA injected animals in regenerated head, trunk and tail pieces.

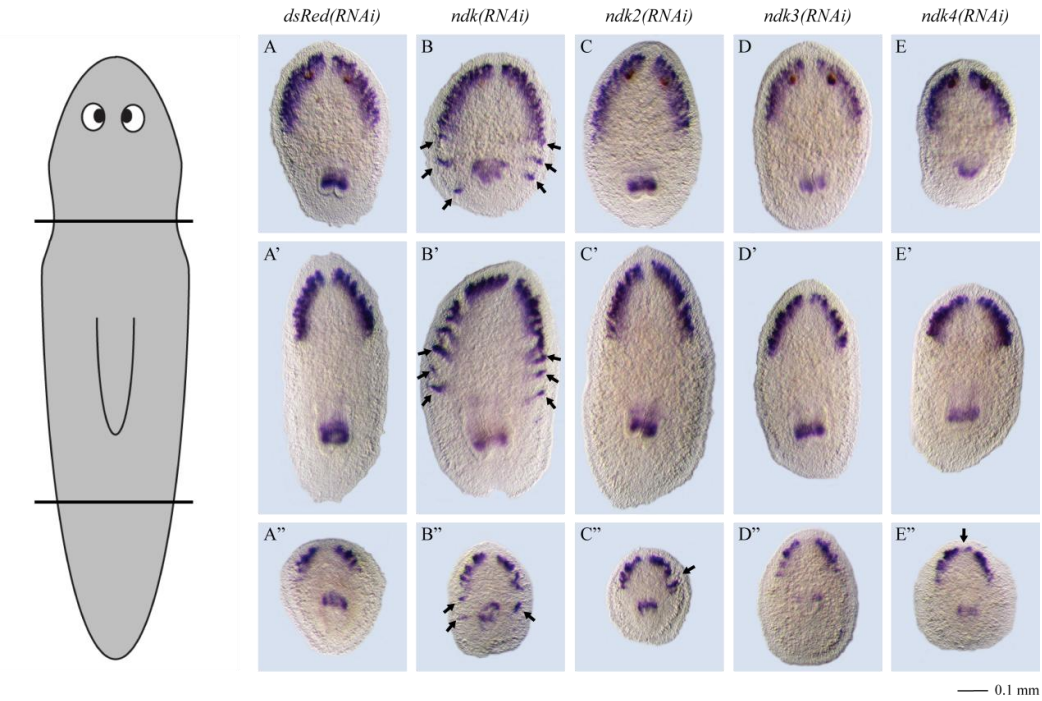


Figure 14: Effect of RNAi of the *ndk* genes after 14 days of regeneration in *S. mediterranea*. A, A' & A": Control animals injected with *dsRed* dsRNA. B, B' & B": *ndk(RNAi)* animals; Brain expansion (indicated with arrows) is observed in the head, trunk and tail pieces. C, C' & C: *ndk2(RNAi)* animals; No brain expansion or other abnormalities are detected in the regenerating head and trunk pieces. The spacing between the branches of the regenerated brain may be increased in the tail pieces of *ndk2* RNAi worms. (indicated with arrow). D, D'& D": *ndk3(RNAi)* animals; The regenerated brains in the head, trunk and tail pieces all resemble those of the control animals. E, E' & E": *ndk4(RNAi)* animals; No brain abnormalities are observed in the heads or trunks. However, joining of both ganglia is detected in the regenerated tail pieces (indicated with arrow) A-E: Regenerated head pieces. A'-E': Regenerated trunk pieces. A"-E": Regenerated tail pieces. Scale bar 0.1 mm.

In pictures **B, G** and **L** of **figure 14**, the brain expansion caused by *ndk(RNAi)* is clearly observed (indicated with arrows). Not only the regenerated brains in the trunk and tail pieces increase in comparison with the *dsRed(RNAi)* animals, but also the already existing brain in the head pieces expand after *ndk(RNAi).* No brain abnormalities are observed in head and trunk pieces in *ndk2(RNAi)*, *ndk3(RNAi)* and *ndk4(RNAi)* planarians. However, in the regenerated tail piece after *ndk2(RNAi)* **(figure 14, M)** larger gaps between the brain branches in comparison to the *dsRed(RNAi)* animals were detected. Also in the *ndk4(RNAi)* tail piece a brain abnormality is observed: the joining of both half's of the brain, which is not visible in the *dsRed(RNAi)* tail piece.

To investigate the possibility that *ndk2* and *ndk4* play important roles during brain regeneration, a *ndk2/ndk4(RNAi)* is performed on animals of *S. mediterranea*, looking for (brain) malformation after 14 days of regeneration.

Pictured in **figure 15 (C-C")**, no brain abnormalities are observed in neither regenerating head, trunk nor tail pieces after *ndk2/ndk4(RNAi)*. *dsRed(RNAi)* animals were used as a negative control **(figure 15, A-A")**, while in *ndk(RNAi)* animals brain expansion was clearly visible **(figure 15, B-B")**. Although an increase in the spacing between brain ganglia and joining of both ganglia may have been detected in regenerated tail pieces of *ndk2(RNAi)* and *ndk4(RNAi)* animals respectively, no malformations were observed in the double knock-out animals. Also no brain expansion was caused by *ndk2/ndk4(RNAi).*

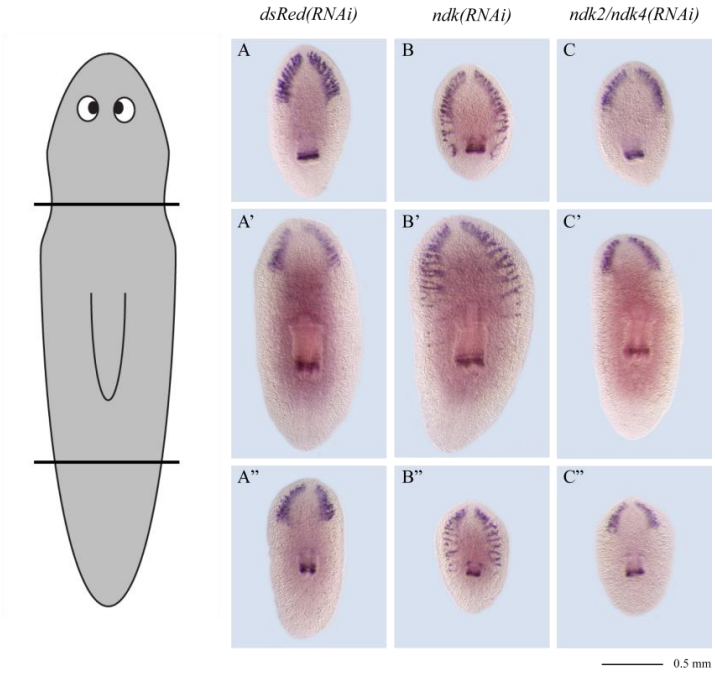


Figure 15: The effects of *ndk2/ndk4(RNAi)* after 14 days of regeneration. A-A": Control animals injected with *dsRed* dsRNA. B-B": *ndk(RNAi)* animals; Brain expansion is clearly visible in both regenerating brains of the trunk and tail pieces and in the pre-existing brain in the regenerating head piece. C-C": *ndk2/ndk4(RNAi)* animals; no brain abnormalities are observed in neither head, trunk nor tail pieces. A-C: Regenerating head pieces; A'-C': Regenerating trunk pieces; A"-C": Regenerating tail pieces. Scale bar: 0.5 mm.

2.3. The effect of **ndk(RNAi)** *on neoblast proliferation*

To investigate other possible roles of *ndk* in *Schmidtea mediterranea,* an anti-phospho histone H3 immunostaining was performed to study the effect of *ndk(RNAi)* on stem cell proliferation. Since FGFRL1 inhibits cell proliferation and promotes cell differentiation, it is possible that knock-out of *ndk* induces an increase in mitoses in planarians. Wenemoser & Reddien (2010) [38] describe the effect of epidermal disruption and amputation on cell proliferation. A small increase in mitotically active cells is detected 6-10 hours after amputation, followed by a decrease in proliferation at 18 hours after amputation and a larger mitotic peak after 48 hours. When the epidermis is disrupted but no amputation is performed, only the first mitotic peak is observed. The possible effects of *ndk(RNAi)* on neoblast proliferation were investigated in intact animals and at the above mentioned time points during regeneration **(figure 16).**

Figure 16 A represents the average numbers of mitoses/mm² in intact control and *ndk(RNAi)* animals. In *dsRed(RNAi)* worms, 254.6 mitoses/mm² are detected while in *ndk(RNAi)* animals 328.6 mitoses/mm² are counted. These data illustrate a increase in the number of cell proliferations in intact *ndk(RNAi)* animals compared to control *dsRed(RNAi)* animals ($p = 0.0928$).

Figures 16 B-D show the effects of *ndk(RNAi)* on regenerating head, trunk and tail pieces at different time points (8 hours, 18 hours and 48 hours). In the regenerating tail pieces 8 hours after amputation, an increase in mitotic cells/mm² in *ndk(RNAi)* animals was indicated compared to *dsRed(RNAi)* animals ($p = 0.0663$). In *ndk(RNAi)* regenerating tails after 8 hours, the average number of mitoses/mm² is 807.1, while in control worms 394.4 mitoses/mm² were detected. In regenerating heads 48 hours after amputation, a significant increase ($p = 0.0255$) in the number of mitotic cells/mm² was observed in *ndk(RNAi)* animals (465.2 mitoses/mm²) compared to *dsRed(RNAi)* animals (389.9 mitoses/mm²).

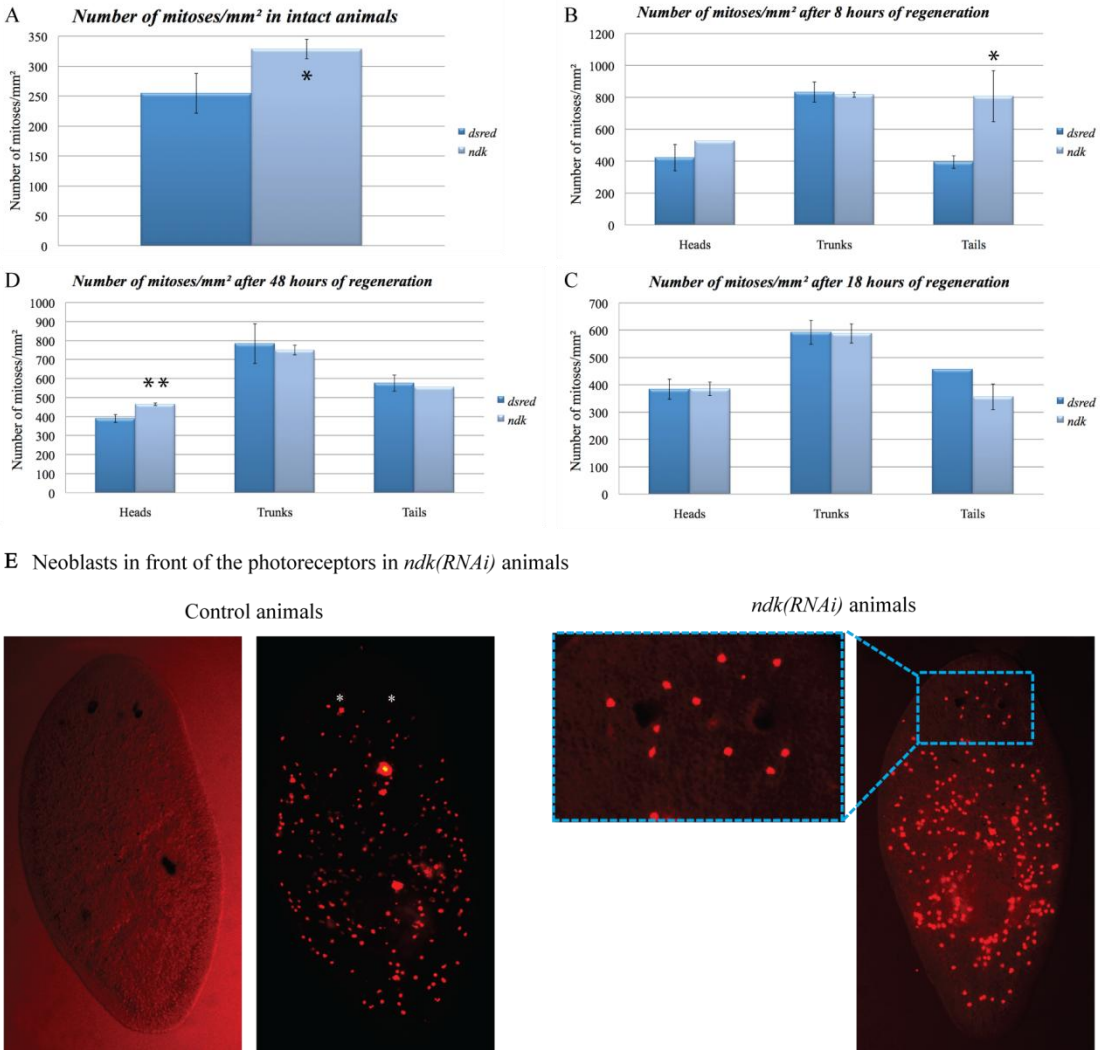


Figure 16: The effect of *ndk(RNAi)* on neoblast proliferation in *S. mediterranea*. A) Number of mitoses/mm² in intact animals. An increase in proliferative cells is detected in *ndk(RNAi)* animals compared to control *dsRed(RNAi)* planarians (p = 0.0928). B) Number of mitoses/mm² after 8 hours of regeneration in head, trunk and tail pieces. A significant increases in mitotic cells is detected in the tail pieces (p = 0.0663). C) Number of mitoses/mm² after 18 hours of regeneration in head, trunk and tail pieces. No significant differences are observed. D) Number of mitoses/mm² after 48 hours of regeneration in head, trunk and tail pieces. In the regenerating head pieces of the ndk(RNAi) animals, a significant increase in the number of mitoses/mm² is detected compared to the control animals (p = 0.0255). E) The presence of neoblasts in front of the photoreceptors in *ndk(RNAi)* animals, in contrast with control *dsRed(RNAi)* animals.

In all intact *ndk(RNAi)* worms (n = 4), the occurrence of mitotic cells in front of the photoreceptors is observed **(figure 16, E).** An average of 4 dividing neoblasts are detected in this region in front of the photoreceptors. Since neoblasts are not normally present in this area in control animals, these mitotically active cells must have migrated to this region in the *ndk(RNAi)* planarians.

IV. Discussion

1. The expression patterns of the ndk *genes*

Thanks to their possession of a population of undifferentiated adult stem cells, planarians have the potential to regenerate complete organisms out of a fragment of tissue. This ability makes these animals ideal modelorganisms to study regenerative capacities and the patterning during regeneration. Whole mount *in situ* hybridizations were performed to study the expression patterns of the *ndk* genes in *Schmidtea mediterranea*. Both intact and regenerating animals were used to provide more information regarding the roles of the *ndk* genes during homeostasis, development and regeneration.

In both intact and regenerating animals, *ndk* is expressed in the brain ganglia and the pharynx. Two independent fixation methods show expression of *ndk* in the pharynx, which suggests that this colour reaction is not the result of a non-specific signal due to unspecific binding of the probe. This is in contrast to the conclusions made by Cebrià et. al. [12] who interpret the signal as non-specific background. The expression patterns of *ndk2* highly resemble those of *ndk*. In intact planarians, *ndk2* expression in the pharynx is only observed using the formaldehyde-based fixation method. In regenerating animals, *ndk2* expression is detected in the regenerating brain ganglia and pharynges, implying that this gene is genuinely expressed in the pharynx. Both fixation processes also result in signal in the pharynx in intact animals for *ndk3* and *ndk4*. During regeneration, expression of these two genes was observed in the regenerating pharynges, similar to *ndk* and *ndk2*.

The similarities observed in the expression patterns of *ndk* and *ndk2* imply that these genes perform similar functions involved in brain development. As all the *ndk* genes are expressed in the pharynx, the possibility exists that *ndk* (and maybe *ndk2*) is not only involved in brain development, but also performs a different function that it has in common with the remaining *ndk* genes.

2. Regeneration after ndk *knock down*

2.1. Ndk(RNAi) *induces formation of early ectopic brain ganglia*

After one week of microinjections followed by 72 hours of regeneration, worms were fixed to study the effect of RNA interference on early regeneration. In the head pieces of *ndk(RNAi)* animals, formation of two ectopic brain ganglia in this posterior blastema were observed. The other *ndk* genes do not have phenotypic effects on the early regeneration. No ectopic brain ganglia were observed in the posterior blastema of the regenerating *ndk(RNAi)* trunk pieces. There are two possible explanations. First, the anterior-posterior gradient of the wnt-signalling [25] may inhibit ectopic brain formation at this posterior position in the animal. Since these ectopic brain ganglia are already very small in the head pieces, the higher wnt-concentration in the more posterior parts of the worms may prevent the ectopic brain formation. A second explanation is that the brain is the source of a ligand with brain inducing activity that is inhibited by binding the NDK protein. When *ndk* is knocked out, this ligand which is still being produced in the brain of the regenerating head piece can diffuse more posteriorly and cause ectopic brain formation.

Hydroxyurea (HU) treatments were performed to identify the neoblast source of these early ectopic brain ganglia. HU blocks the cell cycle at the G1/S transition, so we can determine whether *ndk(RNAi)* induced posterior ectopic early brains form specifically from G2/M neoblasts. *β-catenin(RNAi)* animals are used as a control, since previous experiments performed in the Aboobaker lab have shown that these ectopic brains in *β-catenin(RNAi)* planarians are sensitive to HU and therefore not derived from neoblasts in the G2/M phase [32]. Small ectopic brain ganglia were visible in the *ndk(RNAi)* worms after 48 hours in the regenerating heads. However, significant brain expansion was also detected at this time point 6 days after the initial *ndk* dsRNA injection (3 days of injections, 16 hours HU exposure before cutting and 2 days of regeneration). In light of this observation it is unclear whether the ectopic posterior brain ganglia form specifically in the posterior blastema or whether they form in pre-existing stump tissue. As the ectopic brain tissue could have formed prior to HU exposure it is unclear whether it is truly derived specifically from G2/M neoblasts.

We can conclude that only *ndk* plays a non-redundant role in regulating (early) brain development, since no abnormalities were observed in the *ndk2(RNAi)*, *ndk3(RNAi)* and *ndk4(RNAi)* animals.

2.2. Ndk(RNAi) *animals form posterior brain expansions*

After knock down of the *ndk* genes through RNA interference, brain expansions were observed in *ndk(RNAi)* animals after 14 days of regeneration. These results correlate with the data of Cebrià et al. (2002) [12] and Agata K. & Umesono Y. (2008) [9] on *Dugesia japonica* and are consistent with the capture model proposed by Francesc Cebrià [12]. This model suggests that NDK might function as a decoy receptor that binds FGF ligands and sequesters them away from the conventional FGFRs. Alternative modes to function would be for NDK to accelerate the internalization and degradation of the actively signalling receptors or to recruit other signalling molecules such as tyrosine phosphatases to the sites where the other signalling receptors are located [11]. These are all possible mechanisms through which NDK could function, but more research needs to be done to acquire more information.

No abnormalities were observed in the regenerated head and trunk pieces of *ndk2(RNAi)*, *ndk3(RNAi)* and *ndk4(RNAi)* planarians. In the regenerated tail pieces, brain malformations were detected in *ndk2(RNAi)* and *ndk4(RNAi)* animals compared to the control worm. However, the *dsRed(RNAi)* tail piece did not regenerate a brain with a normal structure. Additionally, only 1 tail piece was analysed in the case of *ndk2* and *ndk4*. Double knock-out experiments were performed to determine whether the detected brain malformation in the *ndk2(RNAi)* and *ndk4(RNAi)* animals are the result of the gene knock out or the regeneration problems. The *ndk2/ndk4(RNAi)* animals did not show any malformations, so we can conclude that nor *ndk2*, nor *ndk4* is involved in brain development.

So far, only *ndk* seems to participate in regulating brain specification, while the role of the other *ndk* genes remains unknown. The possibility exists that the *ndk* genes perform similar functions, since all these genes are also expressed in the pharynx. Trueb et al. [11] lists possible functions of FGFRL1 in different organisms, including *ndk* in planarians. FGFRL1 inhibits cell proliferation and promotes cell differentiation [38]. On the other hand, it also induces cell adhesion and appears to be involved in cell-cell fusion [39]. To unravel the role of the remaining *ndk* genes in *Schmidtea*

mediterranea, these pathways should be investigated as possible targets of *ndk* functioning.

2.3. The effect of ndk(RNAi) *on neoblast proliferation*

In intact planarians, a small increase in the number of mitoses/mm² is observed in *ndk(RNAi)* animals in comparison to *dsRed(RNAi)* worms. Also in the regenerating tail pieces 8 hours after amputation and the head pieces after 48 hours of regeneration, increases in the number of dividing neoblasts in *ndk(RNAi)* animals was detected. These data indicate that *ndk* plays a role in regulating the proliferation of neoblasts in intact and regenerating planarians. Interestingly, the occurrence of mitotic cells in front of the photoreceptors is observed in intact *ndk(RNAi)* animals. Since neoblasts are not normally present in this area, these mitotically active cells must have migrated to this region. A possible explanation is that *nkd(RNAi)* not only induces posterior brain expansions, but also promotes increase in size of the pre-existing brain.

Due limited sample sizes, firm conclusions cannot be drawn from this experiment. In order to conclusively determine the role of *ndk* on cell proliferation, the experiment should be repeated with larger sample sizes. Furthermore, only the effect of *ndk* on stem cell proliferation was examined, while *ndk2*, *ndk3* and *ndk4* may be involved in antiproliferative pathways.

V. Conclusion

Ndk and *ndk2* are expressed in very similar patterns in intact and regenerating animals, showing expression of both genes in the brains and pharynx. Interestingly, all *ndk* genes are expressed in the pharynges, including *ndk3* and *ndk4*, although these genes are not expressed in the brain ganglia. *Ndk(RNAi)* results in the formation of early ectopic brain ganglia and posterior brain expansion. In contrast to *ndk(RNAi)*, inhibitions of the other *ndk* genes do not cause phenotypic malformations. The function of these genes remains unknown and their expression in the pharynx might provide crucial information about their role in the planarian *Schmidtea mediterranea.* Similar to FGFRL1 in multiple organisms, *ndk* seems to be involved in controlling stem cell proliferation in planarians. The possibility exists that the remaining *ndk* genes share this function.

Planarians are ideal organisms to study brain regeneration and development. The functioning of FGFRL genes is studied in multiple organisms, but much remains unknown about the roles and signalling pathways of the *ndk* genes in planarians. Only a few answers have been revealed and additional research is necessary to obtain crucial information about the *nou-darake* genes and their roles in regeneration and homeostasis.

Investigation of octanol treatments as a way to study gap-junctional communication in *Schmidtea mediterranea*

III. Results

Oviedo et al. (2010) [28] shows that long range signalling through gap junctions (GJ) plays a critical role during regeneration in *Dugesia japonica.* Through exposure to a GJ-blocker (such as octanol) and decapitation, animals form bipolar heads. Since octanol exposure to fragments in which a head is present results in normal regeneration, a long range signal must be sent to indicate the presence of the head. Disruption of the VNCs has the same effect as the octanol exposure, namely the formation of a second posterior head, indicating that this long range signal is transmitted through the VNCs.

In this project, this experiment is repeated not only on *D. japonica*, but also on both *Schmidtea mediterranea* strains and the species *Girardia tigrina*. Animals were cut into four pieces (head piece, trunk piece, post-pharyngeal piece, tail piece) and exposed to a range of octanol concentrations for three days. Interestingly, phenotypes suggesting disruption of anterior-posterior polarity, including formation of bipolar heads, are only observed in *D. japonica* **(figure 17)**. Although the animals of the *S. mediterranea* and *G. tigrina* species were exposed to a similar range of octanol solutions, no critical malformations were detected. **Figure 18** shows four graphs illustrating the effect of the different octanol concentration on the three planarian species. For both *S. mediterranea* strains, most deaths occur in the post-pharyngeal and tail pieces.

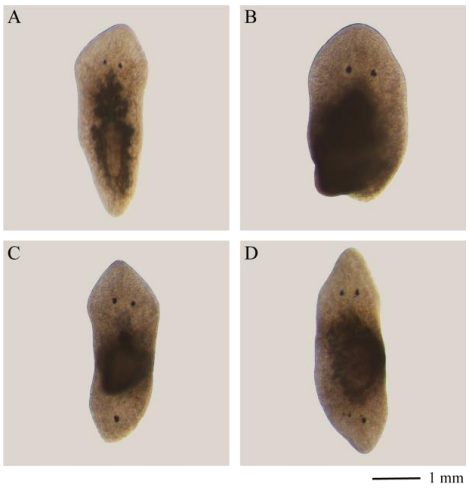


Figure 17: Phenotypes in *Dugesia japonica* after octanol exposure. A) Control animals in planarian water; B) Failure to regenerate; C) Bipolar head formation with only one eye; D) Bipolar head formation. Animals were exposed to a range of octanol concentrations: 0 µM, 25 µM, 50 µM, 75 µM, 100 µM, 127 µM and 150 µM. Scale bar: 1 mm.

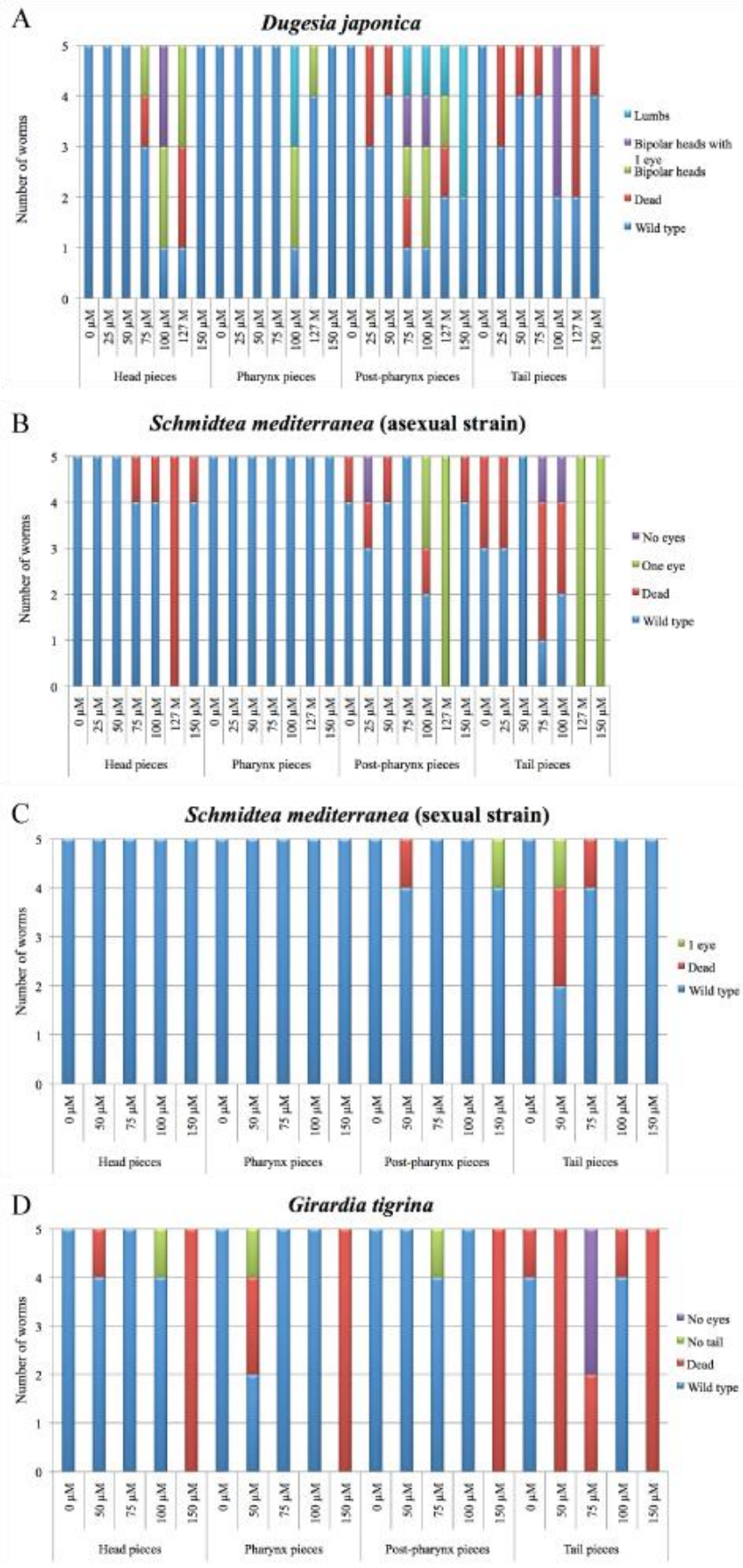


Figure 18: The phenotypic effects of octanol exposure in the planarian species *Dugesia japonica, Schmidtea mediterranea* (both sexual and asexual strains) and *Girardia tigrina*. A) The effects of a range of octanol concentrations (0 µM, 25 µM, 50 µM, 75 µM, 100 µM, 127 µM and 150 µM) on head, pharynx, post-pharyngeal and tail pieces of *D. japonica*. Lumps formed in the pharynx and post-pharyngeal pieces at a concentration of 100 µM and higher. Also formation of bipolar heads (with one or two eyes) was observed in head, post-pharyngeal and tail pieces at 75 µM or 100 µM octanol. B) The effects of a range of octanol concentrations (0 µM, 25 µM, 50 µM, 75 µM, 100 µM, 127 µM and 150 µM) on head, pharynx, post-pharyngeal and tail pieces of the asexual strain of *S. mediterranea*. In post-pharyngeal and tail pieces, some of the animals did not regenerate one or both photoreceptors. C) The effects of a range of octanol concentrations (0 µM, 50 µM, 75 µM, 100 µM, and 150 µM) on head, pharynx, post-pharyngeal and tail pieces of the sexual strain of *S. mediterranea*. In post-pharyngeal and tail pieces, some of the animals only regenerated one eye. D) The effects of a range of octanol concentrations (0 µM, 50 µM, 75 µM, 100 µM and 150 µM) on head, pharynx, post-pharyngeal and tail pieces of *G. tigrina*. All pieces died at 150 µM. Some deaths occured at lower concentrations. Some pieces did not regenerate a tail, while some tail pieces did not regenerate any photoreceptors.

This may be the result of the small size of these pieces. However, most of the malformations, including the absence of photoreceptors or presence of just one of the photoreceptors, also occur in these posterior pieces. These regenerating pieces do not show anteriorisation of posterior blastemas, which is the case in *D. japonica*. In *G. tigrina*, similar abnormalities are detected to those seen in *S. mediterranea*. Some of the tail pieces do not regenerate the photoreceptors and some of the head, trunk and post-pharyngeal pieces do not regenerate a tail. Comparable to *S. mediterranea*, none of these malformations indicate anteriorisation of the animals. However, more deaths are observed in *G. tigrina* compared to both *S. mediterranea* and *D. japonica*. This may indicate that *G. tigrina* is more sensitive to octanol than the other two planarian species.

Discussion

Long range signalling using gap-juctional communication plays crucial roles in the maintenance of the correct anterior-posterior polarity during regeneration. Octonal treatments offer the possibility to study the importance of GJ signalling by blocking the innexins. Oviedo et al. (2010) [28] used this application to study signalling through GJ in the planarian *Dugesia japonica*. In this project, we examined the possibility of using the octanol treatments to study GJ communication in other planarian species, including *Schmidtea mediterranea* and *Girardia tigrina*. Only in *D. japonica*, bipolar head formation was observed. Although the animals of the *S. mediterranea* and *G. tigrina* species were exposed to a similar range of octanol solutions, no critical malformations were detected.

There are three possible explanations for the differences in octanol effects between *D. japonica* and the other two planarian species. First, there is a possibility that *S. mediterranea* and *G. tigrina* do not take up octanol. A possible explanation for this might a difference in mucus density. Planarian mucus consists of complex carbohydrates and a mixture of proteins, so a small alcohol such as octanol can diffuse through a thin mucus layer. It is possible that by creating a more dense mucus layer, *S. mediterranea* and *G. tigrina* can protect themselves against toxic substances. Experiments performed at the University of Hasselt have shown that there are differences in absorption of toxic substances between salt and freshwater living planarians. Saltwater organisms are more sensitive to the absorption of toxic components since these animals do not need to control their osmotic values as precisely in comparison to freshwater organisms. However, all species used in this experiment are freshwater living organisms and the epidermis is highly similar in species belonging to the Tricladida order. Moreover, phenotypic similarity does not implicate that these cells cannot function in different ways. To investigate the absorption of the toxicant by the different planarian species, levels of octanol in planarian tissues should be measured by gas chromatography mass spectrometry [40].

A second possibility of the observed differences in octanol sensitivity is that *S. mediterranea* and *G. tigrina* differ in their ability to metabolize the toxicant. Among n-alcohols, heptanol and octanol are best known for their inhibition of gap juctional

communication (GJC) in both invertebrate and vertebrate animal models [41-51]. In planarians, heptanol or octanol treatments lead to alterations of AP polarity during regeneration, suggesting that the effects of these toxicants on planarian regeneration correlate with their ability to block GJs [27]. However, no research has been performed to investigate the specific mechanism of action and possible metabolism pathways in planarians. Since octanol exposure causes GJC inhibition in various vertebrate and invertebrate models, it is very unlikely that this toxicant does not act on the same mechanism (in this case GJC) in different planarian species belonging to the same Dugesiidae family. But the possibility exists that different metabolism pathways are present in *S. mediterranea* and *G. tigrina* compared to *D. japonica* to avoid octanol toxicity.

A third explanation of the organismal differences is that *S. mediterranea* and *G. tigrina* possess the ability to excrete the toxic substance. Excretion instead of metabolizing the toxicants is an effective way to prevent toxic effects. As mentioned before, although the epidermises of all planarian species are morphologically similar, the possibility exists that epidermal cells of *S. mediterranea* and *G. tigrina* function in different ways compared to *D. japonica.* More research is needed to unravel these questions concerning octanol exposure as a manner to investigate long range signalling through GJ in *S. mediterranea.*

IV. Conclusion

Long range signalling using gap-juctional communication plays crucial roles in the maintenance of the correct anterior-posterior polarity during regeneration. Octonal treatments offer the possibility to study the importance of GJ signalling by blocking the innexins. Oviedo et al. (2010) [28] used this application to study signalling through GJ in the planarian *Dugesia japonica*. In this project, we examined the possibility of using the octanol treatments to study GJ communication in other planarian species, including *Schmidtea mediterranea* and *Girardia tigrina*. Polarity abnormalities, including the formation of bipolar head formation after anterior and posterior amputations, were only observed in *D. japonica*. Species-specific capacities, including a dense mucus layer, metabolizing pathways and excretion abilities, are possible defence mechanisms of *S. mediterranea* and *G. tigrina* against this toxicant. Octanol treatments cannot be used in *S. mediterranea* or *G. tigrina* to study the roles of GJ signalling during regeneration in these planarian species.

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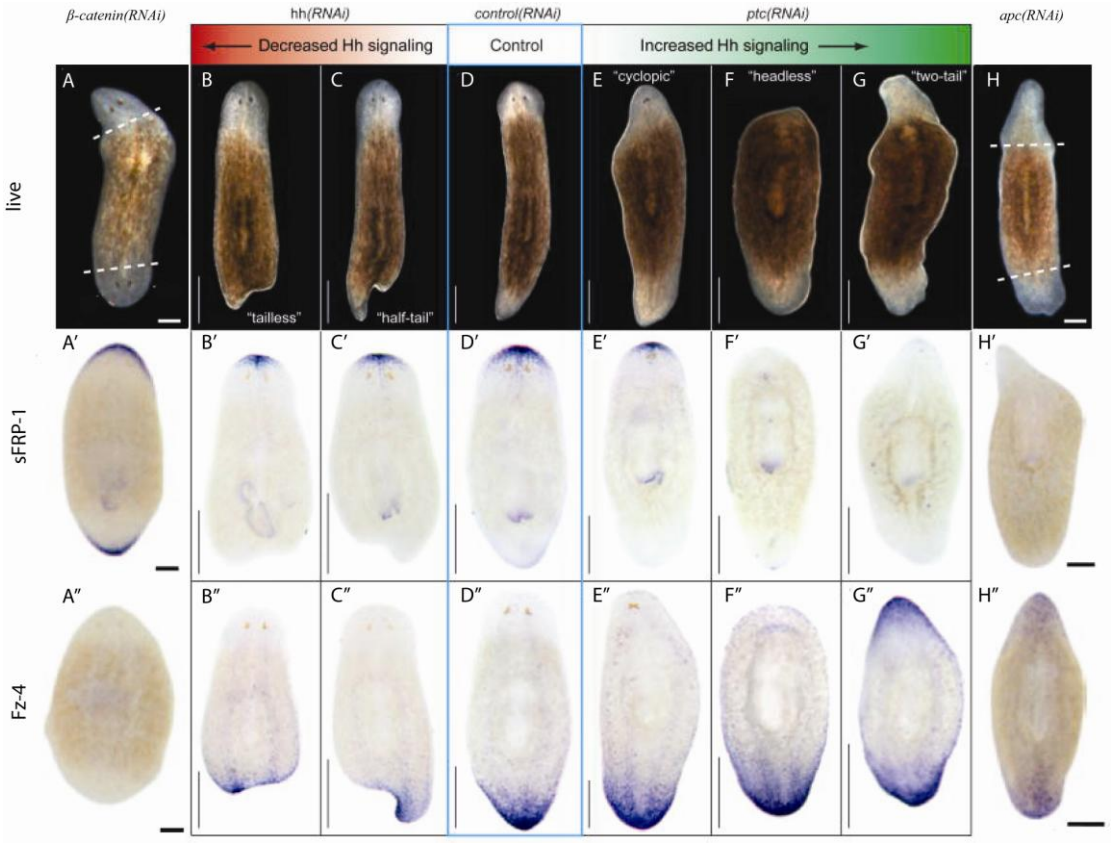
Appendices

Appendix A



A/P patterning defects after RNA interference of various genes. A-H: phenotypical defects; A'-H': sFRP-1 as an anterior marker; A"-H": Fz-4 as a posterior marker. A: *ß-catenin(RNAi)* resulting in formation of bipolar heads. B-C: *Hh(RNAi)* resulting in no-tail or half-tail phenotypes; D: Control animal; E-G: *ptc(RNAi)* resulting in cyclopic, headless and two-tailed organisms; H: *apc(RNAi)* resulting in two-tailed animals. Live images of regenerating trunk fragments 14 days after amputation. Rink et al. (2009) [25]

Appendix B

Material	**Company**
20 x SSC	Invitrogen, , # 15557-036
Acetic acid	Fisher, # A/0400/PB17
Acetic anhydride	Sigma, # 242845
Agarose MP	Roche, # 11388991001
Ampicillin	Sigma, # A9393-5G
Antibody (primary): Rabbit anti-phospho histone H3 mAb	Millipore, # 04-817
Antibody (secondary): Alexa Fluor goat anti-rabbit	Fisher, # VXA11008
Anti-Digoxigenin-AP, fab fragments	Roche, # 11093274910
ATP	Roche, # 11140965001
Blocking reagent	Roche, # 11096176001
Bovine Serum Albumin Fraction V from bovine serum, BSA	Roche, # 10735078001
Calcium Chloride, CaCl2	VWR International, # 100704Y
Chloroform	Medical Stores, # LH-M0067E
CTP	Roche, # 11140922001
dATP	Roche, # 11051440001
dCTP	Roche, # 11051458001
Dextran sulphate	Sigma, # D8906
dGTP	Roche, # 11051466001
DIG-11-UTP	Roche, # 11209256910
DL-Dithiothreitot (DTT)	Fisher, # 32719-0100
dTTP	Roche, # 11051482001
Ethanol	Medical Stores, # LH-M0120E
Formaldehyde	Sigma, # 252549
Formamide	Sigma, # F7503
Glycerol	Sigma, # g5516-1L
Glycogen	Roche, # 10901393001
GTP	Roche, # 11140957001
Heparina	Sigma, # H3393-50KU
Hydrochloric acid, HCl	Medical Stores, # LH-M0165E
Hydrogen peroxide	30% Merck, # 386790
Hydroxyurea	Sigma, # H8627-5G
Isopropyl β-D-thiogalactopyranoside solution, IPTG	Sigma, # I1284
Lithium chloride, LiCl	Fluka, # 62477
Magnesium chloride, MgCl2	Fisher, # M35-500
Maleic Acid	Fisher, # 12523-0010
Methanol	Medical Stores, # LH-M0201E
Micro-injection needles	Drummond Scientific Company, # 3-000-203-G/X
Mineral oil	Fisher, # BPE2629-1
N-acetyl cysteine (NAC)	Sigma,
NBT/BCIP	Roche, # 11681451001

Octanol (1-octanol)	Sigma, # 472328-100ML
Oligo dT primer	Ambion, # AM5730G
Paraformaldehyde, PFA	Fluka, # 76240
Poly(vinyl alcohol), PVA	Sigma, # P8136-250G
Potassium chloride, KCl	Sigma, # P5405
Potassium dihydrogen orthophosphate, KH2PO4	Medical Stores, # LH-M0255E
Proteinase K from Tritirachium album	Sigma, # P4850-5ML
QIA prep miniprep kit	QIAGEN, # 27106
QIAquick Gel extraction kit	QIAGEN, # 28706
Quick ligation kit	NEB, # M22005
RNaseOUT	Invitrogen, # R415
Sodium chloride, NaCl	Fisher, # S/3 160/60
Sodium hydrogencarbonate, NaHCO3	Sigma, # 401676
Sodium hydroxide, NaOH	Fisher, Medical stores, # LH-M0306E
Sodium phosphate dibasic, Na2HPO4	Sigma, # S3264
SP6 RNA polymerase	Roche, # 10810274001
SuperScript III Reverse Transcriptase	Invitrogen, # 18080-044
T7 RNA polymerase	Roche, # 10881775001
Taq polymerase with Thermopol 10 x buffer	NEB, # M0267
Triethanolamine, TEA	Sigma, # 90278
Tris-BASE	Sigma, # T1378
Tris-EDTA (TE) buffer solution, pH 8.0	Fluka, # 93283
Triton X-100	Sigma, # T8787
Trizol reagent	Invitrogen, # 15596-026
Turbo DNase	Ambion, # AM2239
Tween20	Sigma, # P9416
UltraPure (Dnase, Rnase, Protease free) distilled water	Invitrogen, # 10977-049
UTP	Roche, # 11140949001
X-gal	Fisher, # T9554
Yeast tRNA	Ambion, # AM7118

Appendix C

Solution	**Composition**
10 mM dNTP mix	10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP
20 mM rNTP mix	10 mM ATP, 10 mM CTP, 10 mM GTP, 10 mM UTP
10x DIG mix	3.75 mM DIG-UTP, 6.43 mM UTP, 10 mM ATP, 10 mM GTP, 10 mM CTP
5/8 Holtfreter's solution	0.374 M NaCl, 0.416 M KCl, 0.567 mM CaCl2, 1.488 mM NaHCO3 per 1000 ml
Buffer I (fresh)	0.1 % Triton x 100, 11.6 g Maleic acid, 9.76 g NaCl, 95 ml 2N NaOH per 1000 ml
Buffer II (fresh)	1 % blocking reagent, Buffer I
Carnoy solution (fresh)	60 % ethanol, 30 % chloroform, 10 % acetic acid
Hybridization buffer (fresh)	10 % dextran sulphate, prehybridization buffer
PostHyb1 solution (fresh)	50 % formamide, 5 x SSC
PostHyb2 solution (fresh)	75 % PostHyb1 solution, 25 % PostHyb5 solution
PostHyb3 solution (fresh)	50 % PostHyb1 solution, 50 % PostHyb5 solution
PostHyb4 solution (fresh)	25 % PostHyb1 solution, 75 % PostHyb5 solution
PostHyb5 solution (fresh)	2 x SSC, 0.1 % Triton X-100
PostHyb6 solution (fresh)	0.2 x SSC, 0.1 % Triton X-100
Prehybridization buffer	50 % formamide, 5 x SSC, 0.1 mg/ml yeast tRNA, 0.1 mg/ml heparina, 0.1 % Tween20, 10 mM DTT
Reduction solution	25 mM DTT, 1% NP-40, 0.5% SDS in PBS
Resuspension buffer	5 ml formamide, 5 ml 1 x TE pH 7.5, 10 microl 0.1 % Tween20
TMN solution (fresh)	0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 0.05 M MgCl2, 1 % Tween20, 10 % Polyvinyl alcohol
PBS	0.2 g KCl, 8 g NaCl, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4*12H_20$ per 1000 ml

Appendix D

Primer name	**Primer sequence *in situ* probe and dsRNA preparation**
***dsRed* F**	GGCCGCGGCCTCCTCCGAGGACGTCATC
***dsRed* R**	GCCCCGGCGGCCCTCGGCGCG
***Ndk* F**	ATTGTCGTGTGAAGTTAGAG
***Ndk* R**	CGTTGTTGAGGGAGATTT
***Ndk2* F**	GATAGGAGGGCAGAGTATGA
***Ndk2* R**	AAAGCAAAGAGAAGGGGGTAA
***Ndk3* F**	AGGCCTTTCTGTGGAATT
***Ndk3* R**	GTCTGGTTGTGTCGGTTT
***Ndk4* F**	AGTGTACAATCAGTTGGG
***Ndk4* R**	ATAGATTGGGGTTGAGTG
***M13* F**	GTAAAACGACGGCCAG
***M13* R**	CAGGAAACAGCTATGAC

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Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
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