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"THERMAL NOCICEPTION STUDIES IN ZEBRAFISH"

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ABSTRACT

Background

Nociception was defined by Sherrington as the mechanism used to sense painful events. Despite the value of current animal models for nociception and pain, we have not yet understood in full how these mechanisms operate.

Aim

Here we propose a novel approach using zebrafish (*Danio rerio*) larvae in order to determine if the fish is suitable for the study of pain stimuli. Taking advantage of the fact that, in response to noxious stimuli, the expression of several genes in mammals is induced in neurons of the spinal cord and the dorsal root ganglia (DRG), we investigated if such mechanism is evolutionary conserved in the Zebrafish.

Materials and Methods

We cloned homologues of mammals pain marker genes (PMGs) in the fish and we tested their expression after painful stimuli. Thus, we determined an easy and ubiquitous method of noxious stimuli induction in 5dpf larvae by heat shock. We quantitatively assayed these larvae for over-expression of the PMGs by real-time PCRs, to determine if any of these genes is up regulated in specific time points. In another set of experiments we tested, by in situ hybridization, the localization of the PGMs over-expression in the larvae. To confirm the up-regulation of these genes is pain related we looked for the possible heat receptor that could mediate such sensation. Thus, we cloned Zebrafish *trpv1,* analyzed the expression pattern, tested its function with loss of function experiment and by analysis of changes in intracellular calcium levels after various painful stimuli in HEK293T cell line.

Results and Conclusions

These experiments show peculiar over-expression of all the PMGs in time and space, in particular they are expressed in what are probably the spinal cord and the DGR. We cloned and studied *trpv1* as a good candidate for heat sensation mediation. However, zebrafish *trpv1* responds only to low pH when tested in human cell line. Thus, we need further *in vivo* analysis to determine if *trpv1* cooperates with other factors to mediate noxious heat in zebrafish.

INTRODUCTION

Nociception

Nociception is the term generally used to refer to the neural activity of encoding and processing a noxious stimulus, or rather an actually or potentially tissue damaging painful event. In the early nineteen hundred Charles Sherrington identified different types of stimulation leading to painful responses and he called nociceptors the specific neurons mediating these mechanism. Nociception is a modality widely used in the animal kingdom in order to survive in the environment. To confirm and validate the importance of this sensing mechanism, nociceptors have been studied in a wide range of animal species from worms to insects and vertebrates. In mammals nociceptors are located in any area of the body that can feel mechanical, thermal or chemical pain, externally or internally. Cutaneous, cornea and mucosa nociceptors are examples of external mediators, while internal nociceptors are in the organs, such as the muscle, bladder, gut and the digestive track. The primary afferent nociceptors, which detect the noxious stimuli from the body trunk, have their cell bodies in the Dorsal Root Ganglia, DGR. The nociceptors innervating the facial tissue take origin from the Trigeminal ganglia (Tg) and that ones innervating the viscera from the Vagal ganglia (Vg). Both peripheral and central axonal neurons branches innervate their target organs and the spinal cord respectively (Kandel's book). Nociceptors are organized in two classes depending upon their size and level of myelination: A (Aδ and Aβ) and C fibers. Aδ are medium diameter myelinated fibers which mediate acute, well located first or fast pain. They can be divided in of two types: Type I for chemical and high threshold mechanical stimuli and have a heat thresholds >50°C, Type II for a lower chemical but very high mechanical threshold (Basbaum 2009). Aβ are large diameter and rapid conduction fibers, which respond to innocuous mechanical stimuli. C fibers are unmyelinated small diameter for second or slow pain; most of them are polymodal for heat and mechanical stimuli, others are silent nociceptors heat responsive but mechanically insensitive. Primary afferent nociceptive fibers (first order neurons), sending information to the brain, reach the spinal cord where they form synapses in specific laminae using substace P or glutamate as neurotransmitter.

Unmyelinated peptidergic C and myelinate Aδ nociceptors comunicate superficially with large projection neurons in laminae I and interneurons in lamina II. Aδ fibers also arrive in lamina V; unmyelinated non-peptidergic C nociceptors synapse with interneurons in the inner part of lamina II; myelinated Aβ fibers join PCKɣ interneurons in the ventral half of the inner lamina II and in lamina V (Basbaum et all 2009, Jessell 1991). From the specific laminae in the spinal cord the information goes from first order neurons to the second order ones reaching the brain. Some ascending fibers arrive to the somatosensory cortex via the thalamus in order to give informations of the intensity and the location of the stimulus. Other fibers provide information about the affective component of the painful activity engaging the cingulate and insular cortices via parabrachial nucleus and amygdala. The rostral ventral medulla and midbrain periaqueductal gray are the places in which the ascending information arrives to the neurons involved in the descending feedback systems that regulate the output from the spinal cord (Basbaum at al 2009). In These regions of the central nervous system (CNS) the noxious stimuli are elaborated and integrated with other psychological responses becoming what we properly call pain (Figure1).

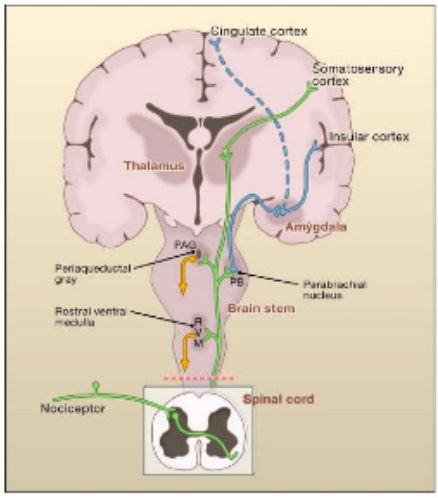

Figure1A

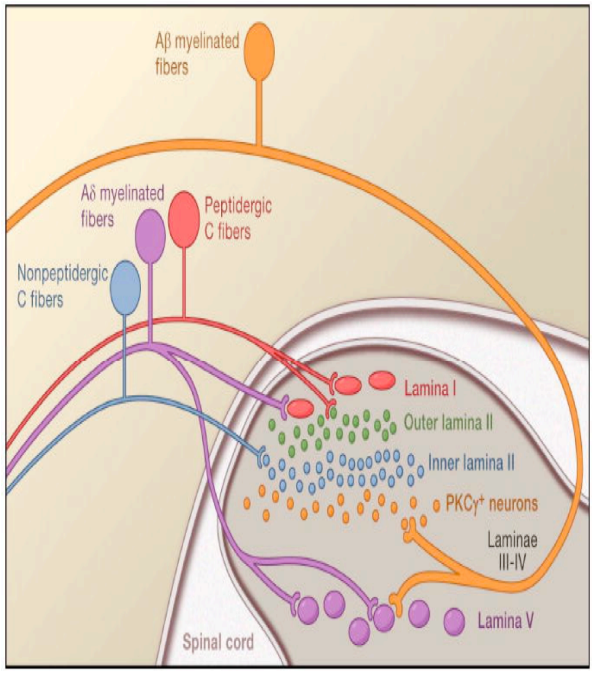

Figure1B

Figure1: pain pathway anatomy (A); Connections between primary afferent fibers and the spinal cord (B) (Basbaum AI, 2009)

To sense noxious stimuli, the nociceptors express different kind of transductional molecules detecting one or more stimulus modalities. The most studied are the transient receptor potential channels, or TRP family receptors; these receptors are tetramers formed by six transmembrane domains and cation-selective pores, usually involved in high calcium permeability (Iatorre 2009) (Figure2).

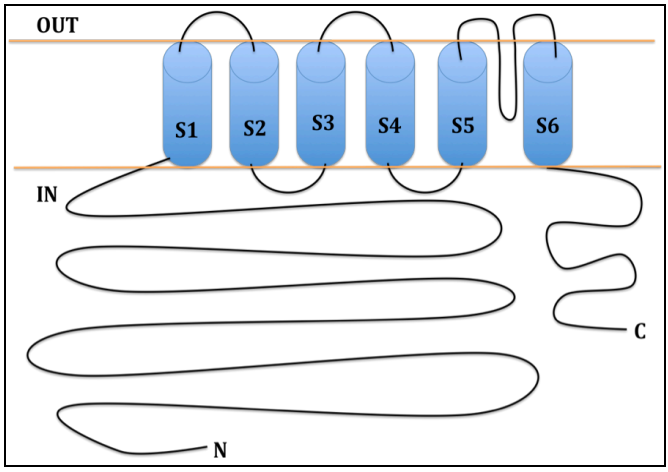

Figure2

Figure2: TRP channel organization. S: subunit, IN: inside the cellular membrane, OUT: outside the cellular membrane, N: amino terminus domain, C: carboxy terminal domain.

TPRs can sense thermal, mechanical and chemical noxious stimuli in organisms ranging from worms to mammals. It appears now clear that each of these receptors has the ability to sense different types of noxious stimuli, working in complexes with other factors, supporting the idea that the nociceptors are polymodal neurons.
Two of the most intriguing TRPs are the Transient Receptor Vanilloid1 and Ankyrin1 (TRPV1 and TRPA1 respectively). The functions of the two of these receptors interlink with each other especially in relation to pain and neurogenic inflammation where TRPV1 is largely co-expressed on the same TRPA1-expressing sensory nerves, in order to integrate together a variety of noxious stimuli (Fernandes 2011). It is well established that TRPA1 detects and mediates behavioral responses to chemical irritants in vitro. The most studied agonist is the mustard oil (the active component being ally1 isothiocyanate) follow by the acrolein (2-propenal) present, for exemple, in tobacco products, byproducts of chemotherapeutic agents and in tear gas.
TRPV1 is activated by vanilloids, including capsaicin, the pungent component of chili peppers. This receptor was first cloned by Caterina in the 1997 and then it was confirmed to be heat > 43°C and pH < 5,9 activated (Tominaga1998). It is localized on small diameter Aδ and C fibre sensory nerves and it is demonstrated to be expressed on neurons throughout the neuroaxis, including such areas as the hypothalamic, hippocampal pyramidal and locus coeruleus neurons, dopaminergic neurons of the substantia nigra in addition to various layers of the cortex (Mezey 2000). The central localization of TRPA1 is less specific than for TRPV1, although TRPA1 mRNA has been shown to be abundant in dog brain and cerebellum (Doihara 2009). Recent studies show that TRPV1 and TRPA1 are also expressed on a multiple of non-neuronal sites vary from vascular smooth muscle to keratinocytes and endothelium (Fernandes 2012).

Zebrafish: new animal model

Generally, rats and mice have been the most used animal models in the field of the pain. Although mice are good candidates for genetic manipulation (Knock out of genes) and a large choice of mutants is already available, however rats have always been the main model system because of the "quasi-human" behavioral responses to stress and pain.
Even if noxious responses have been largely analyzed with complex behavioral techniques (Mogil 2011), the intrinsic molecular and cellular nature of this mechanisms offer also the possibility to work with less evolved animal models species, focusing in the molecular analysis.
Other vertebrates like birds and teleost fish show analogies in the anatomical and molecular structures described previously in mammals. Teleost fish present a central and peripheral nervous system organization very similar, though much simpler, to the mammals. In point of the fact, they show the same types of fibers and the same sensory ganglia (Sneddon LU,).
However, originally there was a controversial argument about fish perception of the pain and it was suggested that in these animals the response to nociception is merely reflexive, thus involving only the spinal cord and hindbrain (Rose 2002).
Further, simple behavioral responses like increasing swimming activity and escaping after noxious stimuli have been studied in teleost larvae (Sneddon LU), suggesting that these animals have proper nociceptive abilities. Moreover, electrical activity of fore- and mid-brain studies suggested different and novel data on telencephalic action in fish after noxious stimuli, demonstrating the possible pain perception in low vertebrates (Dunlop and Laming 2005).

The teleost *Danio rerio* (zebrafish),has been used in the last 25 years as animal model, first of all, because the embryos are transparent and develop externally from the mother permitting in vivo experiments right after the fertilization. This fish as a relatively short regeneration time, reaching sexual maturity in 3 months. The breeding is easy to set up and the fecundity is high: each female can produce 100–200 eggs per mating. The embryos are fast developing so that in 24 hours we can see defined head, eyes, CNS and PNS. Zebrafish have been used for reverse genetics screening, in which the genome of founder fish were randomly mutagenized with ENU or transposons (Mullins, 1993; Talbot WS, Hopkins N. Hopkins 2000). This approaches generated thousands of mutants in the past 15 years and the search is still going.

More recently, direct approaches have been developed to selectively inactivate a gene via injection of antisense modified-oligonucleotides (morpholinos) that can stop mRNA translation (Nasevicius A, Ekker SC ,2000)or by targeting the genome directly with zinc-finger linked FokI endonucleases (Leong IU,2011)
In addition, zebrafish is amenable for *in vivo* studies using the green fluorescent protein (GFP). This protein can be linked to the specific promoter/enhancer of the gene of interest in order to be expressed in the same time and place of the gene. In this way it is possible to visualize and follow the development of the cells expressing the gene of interest. Recently, zebrafish has been demonstrated to be very useful for pharmacological and chemical screening studies: drug administration is possible just dissolving small-molecule compounds in water, where they diffuse into the embryos. Moreover, it was demonstrated that several drugs tested in zebrafish can cause analogous effects to those observed in humans and other mammalian models (Langheinrich U 2003).

Transient receptor potential channels in zebrafish

Many mammalian genes have been cloned in Zebrafish founding out functional similarities. In this contest we can mention several TRP family members.
In mutant zebrafish behavioral studies on TRPA1a and TRPA1b paralogues show that TRPB1 is necessary for behavioral responses to chemical irritants. However, zebrafish TRPA1 paralogues do not response to temperature changes and are not necessary for mechanosensory hair cell function in the inner ear or later line. These results suggest a role for Zebrafish TRPA1 in chemical but not mechanical or thermal sensing (Prober et al. 2008).
The gene coding for TRPV1 has been cloned in Zebrafish (Caron et al 2009) but this report shows only that it is present in a sub-population of the zebrafish trigeminal ganglia.
TRPV4 is one of the mediator for osmotic changes and it has ,in different organisms, a central role in osmoregulatory responses. Steve Mangos (2006) reported *trpv4* expression in different organs of the fish during the development. He observed *trpv4* mRNA expression in 4-cells embryos and 1 somite stage during the notocord formation until the 24hpf when there is also a large expression in the brain.

Trpv4 is also present in in the pronephric kidney mostly in the distal nephron segment and in the cloaca around 32–48 hpf. In the same moment the receptor is espressed at the level of the primarily hair cells of the lateral line organs.
The expression of trpv4 in, brain, kidney, heart and lateral line organs persists at 72hpf while expression in the notochord is down-regulated.

Pain marker genes

In mammals, in response to peripheral nerve injury, synthesis and translation of several genes are induced in specific neurons. Some of the factors synthesized are PACAP (Pituitary adenylate cyclase-activating polypeptide), VIP (vasoactive intestinal peptide), BDNF (Brain-derived neurotrophic factor), c-Jun (Ru-Rong Ji and Strichartz 2004). Moreover, one of the most ubiquitous and intriguing molecular marker is the oncogene *c-fos* which expression results to be induced in the mammal spinal dorsal horn neurons within 0.5/2.0 hours after lesions occur. The spinal cord neurons, where *c-fos* expression is up-regulated, are between the direct source of signal (peripheral nerves) and the central area of the brain where these signals are finally elaborated (Joachim S 2007). In fish Thiesse et al (2001) showed the expression of *c-fos* in vast areas of the CNS and PNS.
PACAP is sintetized from adcyap1a gene (ZFIN) and it has a wide range of function as a neuromodulator, neurotropin, smooth musce relaxant (Fradinger EA, Krueckl SL 2003); PACAP can also stimulate the growth hormone release from the pituitary cells (Parker 1997, Wong AO 1998). In Human there are two biologically active gene forms, PACAP27 and PACAP38, where the second one is a C-terminal extension of PACAP27 (Miyata et al. 1989, 1990). In the fish two *pacap* genes were identified and the gene encoding the peptides has been duplicated producing two forms of each peptide (Fradinger & Sherwood 2000, Wang et al. 2003). In particular, adcyap1b is expressed on the telencephalon, the diencephalon, the rhombencephalon, the neurons in the dorsal part of the spinal cord and appears to be the most abundant during brain development (Alexandre and al. 2011). VIP is a potent peptide that has many functions such as stimulates exocrine and endocrine secretion, moderate smooth muscle relaxation, regulate circadian rhythms and neuro-modulation (Tse 2002).

Vip expression in fish is showed in the enteric nervous system (Van Nassauw L 2010) and hindbrain, midbrain and mesencephalon (Wu at all 2008). BDNF is a neurotrophin (brain-derived neurotrophic factor) that supports the survival of existing neurons and stimulates the differentiation and the growth of new neurons. *bdnf* gene is strongly expressed on the fish brain (Thisse et al., 2004). *c-jun* is the putative transforming gene of avian sarcoma virus 17. Its product may dimerize with C-Fos in order to form the AP-1 transcription factor, which up-regulates the transcription of several genes involved in differentiation and proliferation, regulating the defense against bacteria and virus invasions and cell damages. In fish, Gonzalez et al (2006), showed the expression of *c-jun* in the brain, gill, liver and muscular system.

AIMS OF THE THESIS

The goals of this project are the establishment and standardization of a noxious thermal test in zebrafish larvae. In response to the thermal nociceptive stimulus we will analyze the expression of a set of genes that we called pain marker genes, PMGs. These genes are induced by various nociceptive stimuli in the mammals, thus our final aim is to demonstrate if the mechanism that regulate the expression of the PMG in response to noxious heat is evolutionary conserved between mammals and teleosts.

MATERIALS AND METHOD

-Fish Husbandry

We used zebrafish from *Brass* strain, which carries a mutation affecting the pigmentation, in order to have a lighter stain. This fish line is generally accepted and used as *Wild type* fish. Animals were maintained in a 14h/10h light/dark cycle following standard husbandry protocols (The Zebrafish book, Monte Westerfield). Embryos were obtained by natural mating in mating cages and collected in Petri dishes with embryos water. They were cultured at 28.5°C in the incubator, with 1x phenylthiourea (PTU) in embryo medium to stop the formation of pigment. The developmental stages of the embryos were determined days post fertilization (dpf). Animals were handled according to the guidelines of IACUC, which are the internal Temple University rules to work with this animal model. Every effort was made to minimize the number of zebrafish used.

-Heat shock nociception test

We used little plastic baskets to dip 10 embryos of 5dpf, for 5 seconds, in water at increasing temperature steps. Starting from 35°C of temperature and increasing 5°C each time we determined the highest temperature to set up the experiment, without killing the animals, at 48°C. Thus, we analyzed the expression of the PMGs, after the thermal nociceptive stimulus, at different fixed time points: T30, T2h, T4h, T6h, T24h; we considered 5dpf and 6dpf embryos like negative controls.

For each time point samples were fixed in 4% paraformaldehyde/PBS (PFA) at 4^0C overnight and then frosted with methanol at -20°C at least for 2 hours.

-RNA extraction

Total RNA was extracted using Quiagen RNeasy Mini Kit.

We used 10 embryos at 5dpf for each PMG. We cleaned up the embryos twice with PBS and we disrupted them with specific smashers in a 1.5ml tube. We mixed the sample with 600 μl of RTL buffer from the kit and 6μl of β-mercaptoethanol in order to have a complete homogenization. Thus, we added 1 volume of 70% ethanol to the lysate and mixed by pipetting. After centrifugation of the sample in a RNAeasy Mini spin column we went on with centrifugations, first with 700μl of RW1 buffer and twice with 500μl of RPE buffer. RNA was eluted from the column with 30 μl of RNase-free water. We incubated RNA for 30 minutes at 37°C with Promega DNaseQ in order to eliminate DNA contamination. RNA concentration was detected reading the absorbance at 260 nm with a spectrophotometer (Nanodrop from Roche).

-cDNA synthesis

Total RNA (1μg) was reverse transcribed to cDNA using the SuperScript® III First-Strand Synthesis System kit. The RNA was combined with 10 mM dNTP mix and Random Hexamers (50ng/ul) in a 10μl mixture. It was incubated at 65°C for 5 minutes and placed on ice. The RNA/primers mixture was added to 9μl of 10X RT buffer, 25mM MgCl2, 0.1 M DDT and RNaseOUT (40U/μl) reaction mix and incubated for 2 minutes at RT. Then, 1μl of SuperScript III RT was added and incubated in the reaction for 10 minutes at RT, 50 minutes at 42°C, 15 minutes at 70°C terminating on chilling on ice.

-PCRs

We designed gene specific primers for each PGM and trpv1 cloning, and trpv1 loss of function experiment (Table1).

CLONING PRIMERS	5'SEQUENCE	3'SEQUENCE	FRAGMENT SIZE
c-Fos	ttgcagtggatggtccagc	aggtagtgacgatctctggg	438bp
c-Jun	agcgatatcctcacttctcc	tttcctcttccggcatttgg	582bp
VIP	aatgcttgtgcggaacggc	aacgtgtcggatccaaggc	677bp
Pacap1b	ataaaagtgaaggggttgaaccg	acaaataagcaaatcgacgtcc	683bp
BDNF	ttgcatgagagctgcgcc	aaccgccagccgatcttcc	717bp
TRPV1/2A	aagggaacatacctcaagcc	tttggcagcgagtgtgattg	1046bp
TRPV1/2B	aacacagcgttcgtcatcgc	agtcaatccaaatcgtccc	1064bp
TRPV1/2C	agacataccttccctgaacc	aaggacaccttgtagactcc	1162bp
EcorI TRPV1/2	aggaattccgtcactatgagtaaatcaatgg		
XbaI TRPV1		ctagtctagactagtcatcagacggagctgg	2525bp

-**Cloning**

We cloned the RT-PCR products with a TA based cloning into pCRII TOPO, we used TOPO TA Cloning® Kit Dual Promoter (with pCR®II-TOPO® vector) with One Shot® TOP10F´ Chemically Competent E. coli following the kit instructions.

-Plasmid extraction

We extracted plasmids using the Roche High Pure Plasmid Isolation Kit.

-q-PCRs

Up regulation of the PGMs was quantitatively tested by real time PCRs. For each heat shock time point RNA from 10 embryos was extracted and cDNA was prepared as described above. The cDNA concentration was determined by measuring the absorbance at 260nm with a spectrophotometer (Nanodrop from Roche). The quantification of the PCR products was accomplished with a standard curve using the SYBR-Green method. The SYBR-Green was included in a 2X Master Mix from Roche. We used oligonucleotides primers to amplify ~100bp fragments of each PGM using 100ng/μl of specific cDNA in each reaction (Table2)

We texted the same gene in triple and we used *actb* (actin beta) and *gadph* (Glyceraldehyde 3-phosphate dehydrogenase) specific primers as standards.

The final volume of each reaction was 9μl of Master Mix and 1μl of cDNA. The Standard curve was constructed for each PGM by serial dilution of cDNA: 25ng/μl, 50ng/μl, 100ng/μl and 200ng/μl . The amplification reaction took place in a Roche detection system, with the following conditions: pre-incubation 95°C, amplification at 95°C-64°C-72°C for 45 cycles, melting at 95°C-65°C-97°C, cooling at 37°.

Three PCR reactions were performer for each sample per plate and each experiment was repeated three times: 2 times from 2 different preparations of cDNAs from the same RNA, 1 time from a new RNA. We analyze the results using the second derivative test

q-PCR PRIMERS	5'SEQUENCE	3'SEQUENCE	FRAGMENT SIZE
c-fos	atctgagtgtctaagtgcc	tgcttctcttgttggaggtc	190bp
c-jun	attaaagccggaggaagcg	actttctgcttgagctgtgc	180bp
vip	ggctcttcacaagcggatac	atcatcactgacccgctttc	92bp
pacap1b	cacgcctattgggatgactt	caaaagccaggtcgcttaac	89bp
bdnf	gagctcagcgtttgtgacag	gtctggcccgacatgtctat	80bp

Table2

Probe preparation reaction

For probes synthesis, we linearized the plasmids with restriction enzymes and used the appropriate RNA polymerase as indicated in Table3.

PLASMID NAME	RESTRICTION ENZYME	RNA POLYMERASE
pCRIITOPO - c-fos	EcorV	Sp6
pCRIITOPO - c-jun	NotI	Sp6
pCRIITOPO - vip	EcorV	Sp6
pCRIITOPO - pacap1b	EcorV	Sp6
pCRIITOPO - bdnf	EcorV	Sp6

Table3

The antisense Digoxygenin-labeled probe reaction for each PGM, using the appropriate RNA polymerase, was incubated for 2-3h at 37^0C. Then it was mixed with a 20mM Tris/HCl pH 7.5, 20mM EDTA, 100mM NaCl TEN buffer and purified through centrifugations for 3' at 3000 rpm in a probe QuantG50 Micro column (Biolab), previously balanced with 0.3M NaOAc (pH5.2), 0.1% SDS equilibration buffer. After precipitation with 3M sodium acetate the dry probe was dissolved in 30μl of DEPC H20.

-*In situ* hybridization

Samples were rehydrated by rinsing in an increasing dilution of methanol/PBS and permeabilized with Proteinase K (10μg/ml) for 10-30 minutes at room temperature. The embryos were re-fixed with 4% PFA and then washed with PBT (PBS+Tween-20). Subsequently they were prehybridized for 2 hours with hybridization solution containing 60% formamide (Fisher). The digoxigenin-labeled probes were applied to the respective samples and hybridization was carried out overnight at 70° C. Embryos were washed at decreasing formamide and citrate buffer (SSC) solutions and then 1 x PBT to remove unbound probe. The solution was replaced with antibodies against DIG conjugated to alkaline phosphatase (AP), diluted 1:200. The 2 hours incubation was followed by 8 1xPBT washes, 15 minutes each. PBT was then substituted with AP buffer (100mM TrisHCl pH9.5, 50nM MgCl2, 100mM NaCl, 0.2% Tween 20 and 0.2% Triton-X100) that was applied in 3 washes, 5 minutes each. The DIG-AP antibody was detected with BM purple substrate (Roche) kept in the dark at RT with little agitation for coloration. The reaction was stopped (generally after 1 day) with PBS buffer washes and then fixed with 4% PFA. For longer preservation the embryos were kept in 75%glycerol in 1xPBT. The images were captured with the microscope and analyzed.

-HEK-293Tculture

Cells were seeded in 100mm plates in 10 ml DMEM medium (CellGro, Mediatech Inc., Herndon, VA) with 10 %FBS (DMEM complete) and incubated at 37^0C with 5%CO_2. Cells were split in new plate every 3-4 days, they were first rinsed with sterile 1x PBS then incubated with 2mL of DMEM –Tripsin-EDTA (tripsin concentration vvvv) for ~3min at 37^0C. Tripsin activity was stopped adding 8ml of complete DMEM and 2mL of this mix was used to plate in a new dish with other 10mL of fresh complete DMEM.

-Cells transfection

The day before transfection the cells were seeded in a 96 wells plate at ~30% confluency using Optimem + GlutaMax and 5% FBS as medium. The following day each well was transfected using Lipofectamine 2000 (Invitrogen) with 200ng of pCS2- Flag-trpv1 or 200ng of pCS2- Flag for 18 hours.

-Cells immunoistochemistry

Cells were fixed for 20 min in 4%PFA and then permeabilized for 10min with 0.1% Triton X-100. After 3x washes the cells were placed in blocking solution (1% BSA in 1xPBS) 1h at room temperature and then incubated overnight at 4^0C with anti-Flag antibody (Rockland) after a dilution 1/100 in blocking solution. The cells were incubated with a goat anti mouse-FITC secondary antibody for 1h, at room temperature, in blocking solution. The cells nuclei were stained with DAPI in 1x PBS for 1min. After washes cells were visualized with Olympus IX81 fluorescence microscope (Olympus Microscopes, Center Valley, PA), using a 20X magnification objective.

-Knock down of *trpv1*

We performed hand made microinjections of 1-2pL/embryo of 0.5mM, 1mM and 2mM antisense morpholino against the ATG of trpv1, in 2-4 cells embryos, to test the highest concentration of morpholino that can be used in our experiments without kill the embryos. After 3-4-5dpf we achieved RNA extraction and cDNA synthesis from the microinjected fish in order to detect the *trpv1* expression using PCR approach (Table4).

PRIMERS TO TEST MORPHOLINO	3' SEQUENCE	5' SEQUENCE
exon2 *trpv1*		tgacgtggtggaaccctctagc
*trpv1*A	tttggcagcgagtgtgattg	

Table4

RESULTS

Noxious heat test

We decided to test the noxious effect of high temperature in zebrafish larvae due to the simplicity of the test (Figure4) and the analysis of Prober et al. 2009, that confirmed the ability of zebrafish larvae to sense water temperatures >36^{o}C or <16^{o}C by showing a behavioral response (increasing in locomotor activity). We choose to test 5 to 6dpf larvae because that is the stage in which the larval fish begins autonomous feeding and usually starts to swim in search of food in its natural environment. According to these reasons we postulated that, by 5dpf, the larvae should have the ability to sense and respond to noxious stimuli.

Batches of 10-20 embryos were pooled in small baskets, closed at the bottom with a fine mash net, and immersed for 5 seconds into a beaker containing embryo medium. To determine the optimal temperature inducing the noxious stimulus to the larvae we did incubate the embryos at increasing temperatures, starting from 35°C and increasing 5°C each time. Thus, we determined the highest temperature to set up the experiment, without killing the animals, at 48°C.

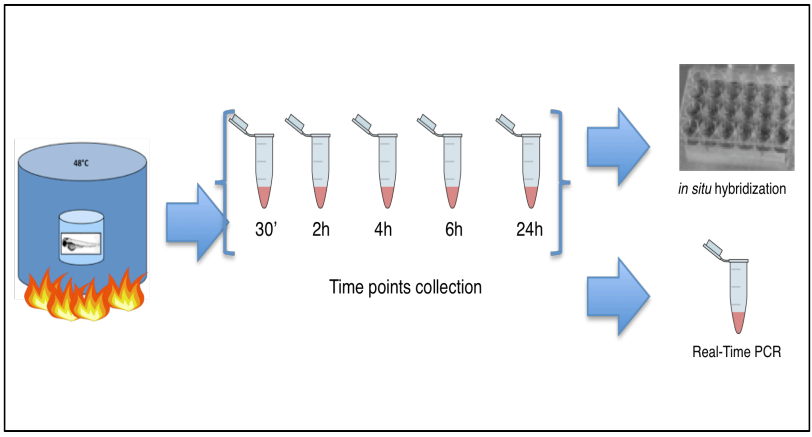

Figure4

Figure4: 10/20 embryos of 5dpf were dipped, for 5 seconds, in water at 48°C. Embryos were fixed in 4%PFA at several time points after the heat shock stimulus: T30min, T2h, T4h, T6h, T24h; 5dpf and 6dpf embryos were considered like negative controls.

Quantitative modification in Pain Marker Genes expression after noxious heat

It is well known that the expression of some genes is induced after noxious stimuli and pain. For example, *c-fos* is induced in neurons of the dorsal horn of the spinal cord and in the DRG (Coggeshall 2005). After neuropathic pain induction many other factors are also activated in DRG (Ru-Rong Ji and Gary Strichartz, 2004). Thus, we chose to study in 5dpf larvae the effect of the noxious heat stimulus on the expression of zebrafish *c-fos, c-jun*, *pacap1b*, *vip* and *bdnf* genes. We called these genes all together pain marker genes (PMGs). We quantitatively analyzed if the PMGs were over-expressed after noxious heat stimulus via relative-q-PCR experiments and we examined the results with the second derivative method, choosing 6dpf mRNA expression level to normalize our data. *c-fos* and *c-jun* are early responsive genes and Figure5 and Figure6 presents their fast mRNA expressions followed by a consequential decreasing at the late time points.

As expected*, c-fos* showed the earliest and strongest expression just after 30min from the noxious heat stimulus and it resulted to be activated ~14 folds more than the control sample at 6dpf. In the other time points its expression level appeared close to the level of control (Figure5)

c-jun transcription was also rapidly activated by ~5 folds after 30min of noxious heat stimulus, this initial activation reduced at one half after 2-4hours and to one third after 24hours from the noxious heat stimulus (Figure6).

vip mRNA was expressed after 30min from the noxious heat stimulus by 3.5 folds (+/- 1) and it was still high at 4h after the thermal stimulus; *vip* expression decreased in the sequent time points (Figure7).

pacap1b mRNA was activated also after 30min from the thermal stimulus by ~3.5 folds (+/- 1) but it reached its maximum height at 4h after the thermal stimulus and decreased in the later time points (Figure8).

bdnf mRNA level increased too arriving to its highest point at 4h after the heat stimulus and then came back to the physiological level (Figure9).

These data clearly indicate that the heat shock stimulates the PGMs over-expressions with an early pick response at 30min and that these inductions are time dependent.

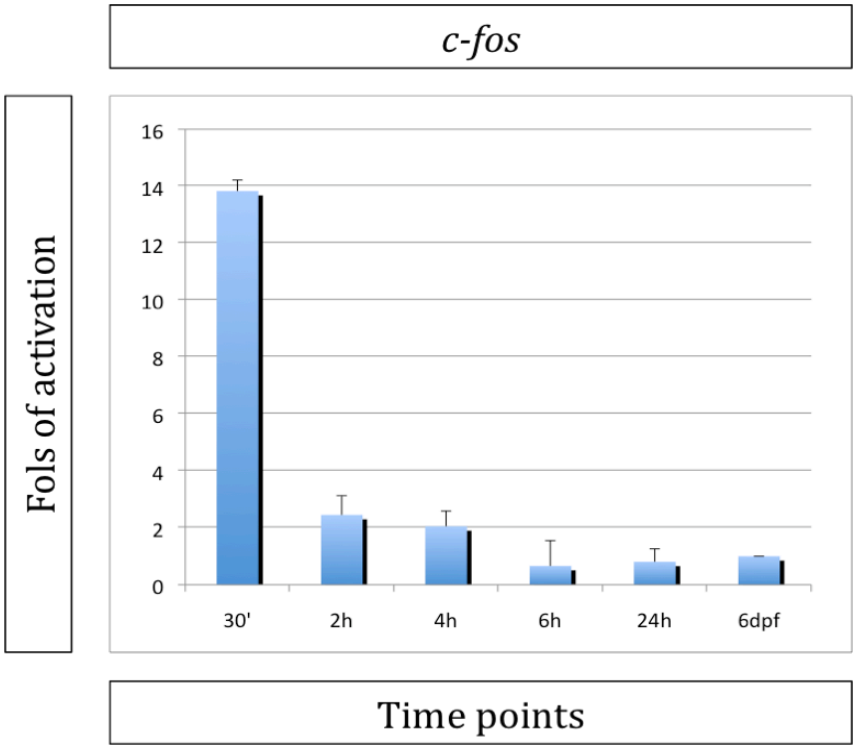

Figure5

Figure5: high level of *c-fos* mRNA at 30' minutes after the heat shock experiment. This quantity critically decreases during the 24h.

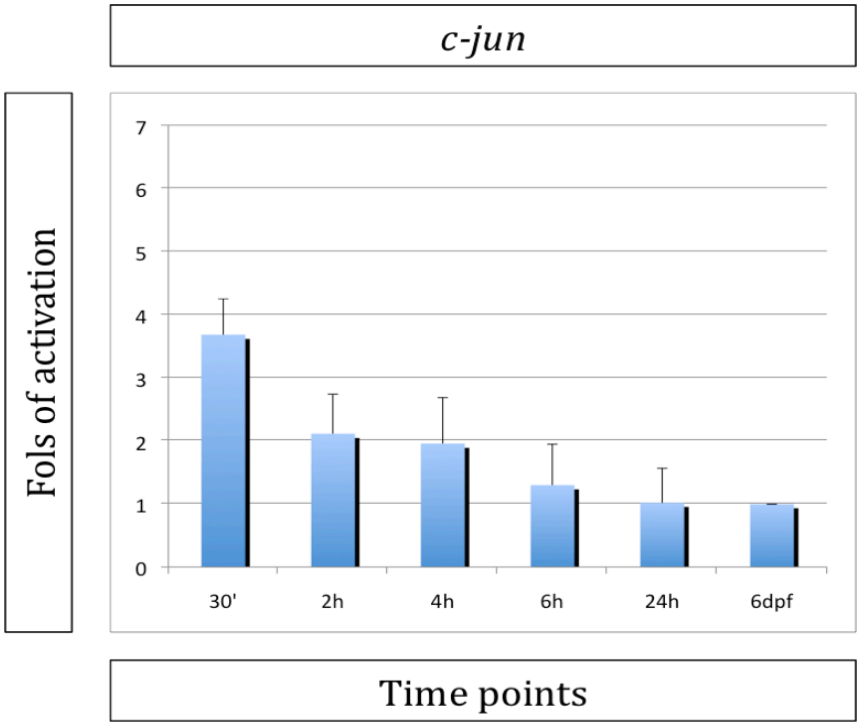

Figure6

Figure6: *c-jun* mRNA expression is aboundant at the 30' time point after the heat shock and decreases within the 24h after the stimulus.

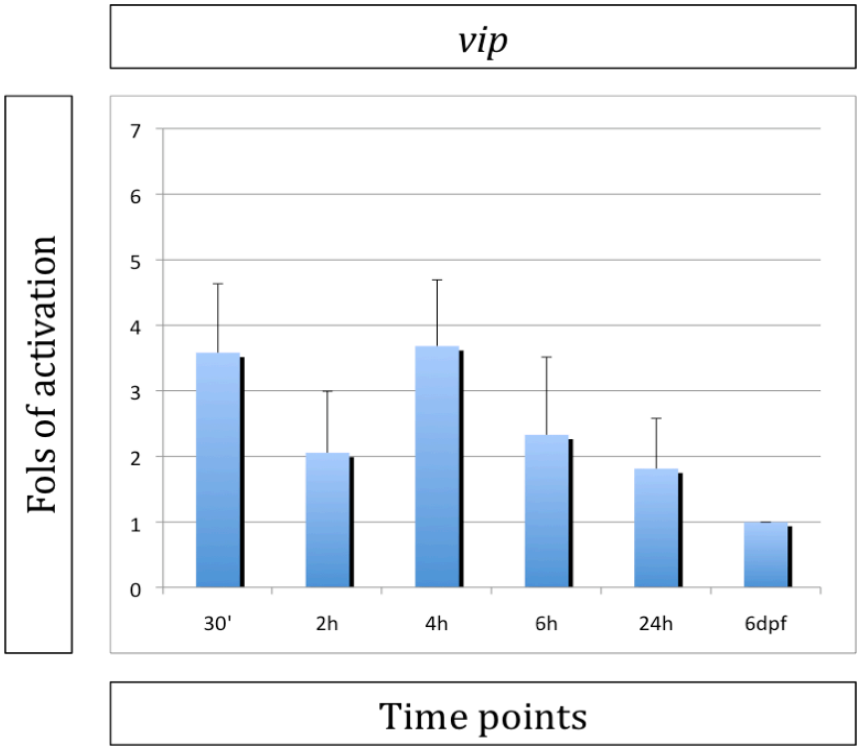

Figure7

Figure7: *vip* mRNA quantitative expression during the heat shock experiment: after 30' there is a high level of *vip* mRNA that remains abundant until the 4h.

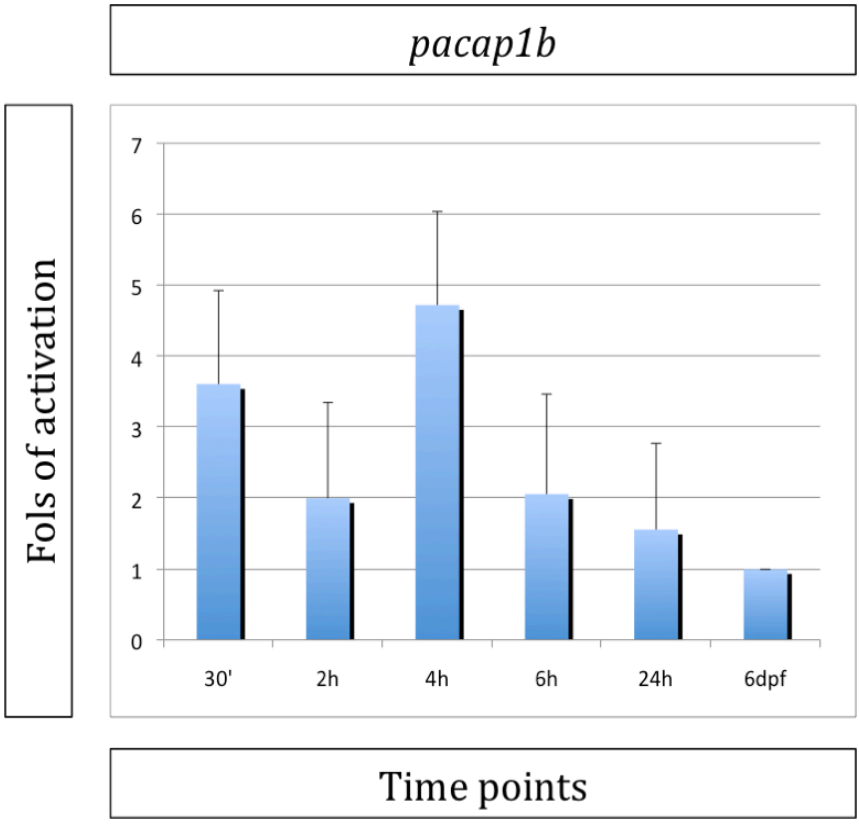

Figure8

Figure8: *pacap1b* mRNA level is mostly abundant after 4h from the thermal stimulus.

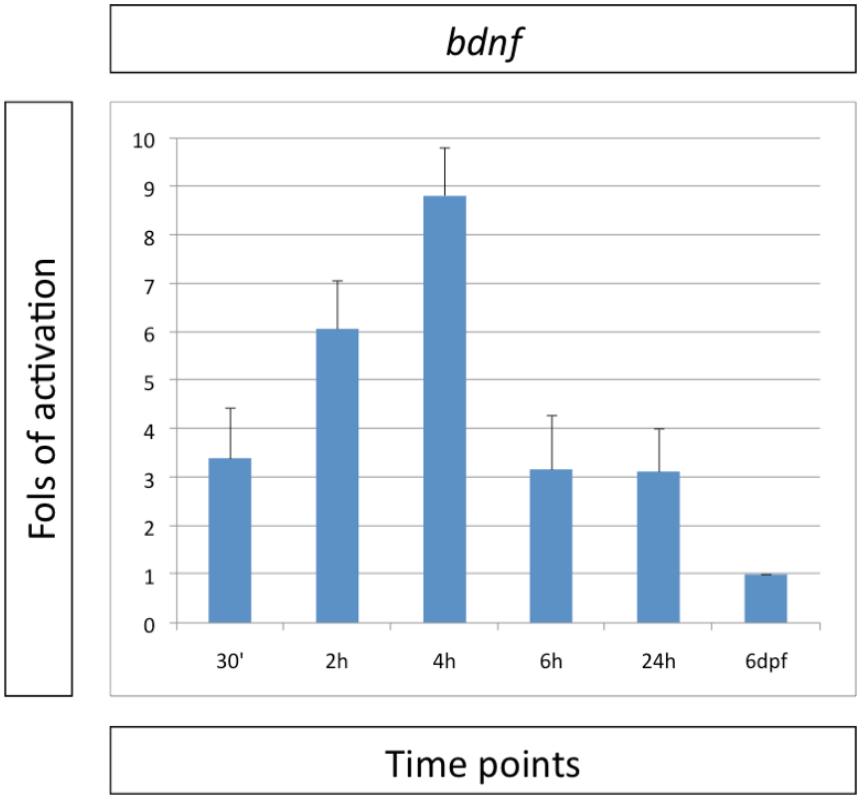

Figure9

Figure9: increasing level of *bdnf* mRNA starting from 30' to a maximum at 4h after the heat shock. As the time goes by *bdnf* quantity decreases in order to come back to the physiological level.

Localization of the PMGs in heat shocked zebrafish larvae

Taking advantage of the fact that, in response to noxious stimuli, the expression of the PGMs in mammals is induced in neurons of the spinal cord and the dorsal root ganglia we performed *in situ* hybridization experiments after heat shock, in order to investigate if this mechanism is evolutionary conserved in the Zebrafish.
Confirming the relative q-PCR we found that, after the thermal nociceptive stimulus, all the PMGs were over-expressed in specific time points. *c-fos* and *c-jun* are early responsive genes and, like we attended, we observed after 30' from the stimulus an immediate over-expression. *bdnf* expression showed the same response to the heat shock having its maximum of expression at 4 hours; *vip* and *pacap1b* showed instead a late comeback to the stimulus, having their higher over-expression level between the 4 and 6 hours. All genes returned to the physiological condition after the 24 hours (table5). Thus, the in situ experiments in 5-6dpf zebrafish larvae confirmed the induction of the PMGs expression in a time fashion similar to that one previously seen in our relative q-PCR analysis .

	5dpf	**30'**	**2h**	**4h**	**6h**	**24h**
c-fos	0/14	14/14	11/11	9/10	10/13	0/14
c-jun	0/12	10/10	8/14	2/14	0/14	0/14
vip	0/15	7/10	10/14	12/14	15/17	4/12
Pacap1b	0/12	5/14	6/13	9/14	11/13	3/15
bdnf	0/12	4/13	6/16	13/16	9/11	0/13

Table5

Table5: ratio between over-expressing embryos and total embryos for each PMG and at each time point.

More in detail, we found that PGMs were over-expressed in specific region of the body relevant for nociception like in the spinal cord, the lateral line neurons, the brain, but also in other regions like epidermis and a region surrounding the gut and the kidney. *c-fos* that is usually expressed at the level of tegumentum, the hindbrain, the trigeminal ganglia, the spinal cord neurons, the telencephalon, the diencephalon and a small population of blood cells (Thiesse et al, 2000), appeared to be over-expressed at the level of the lateral line and the spinal cord and it was more abundant in the brain in respect to the physiological situation 30' after the stimulus (Figure10).
c-jun is normally present in the brain, gill, liver and muscular system (Gonzalez et al, 2006). In our experiment it seemed to be over-expressed at the level of the gut, in which we saw a homogeneous coloration, and also in spots at the level of the lateral line, which could be lateral line neurons at 30' after the stimulus (Figure11). The normal expression of *vip* is in hindbrain, midbrain and mesencephalon (Tse, 2002); after the heat noxious stimulus there was clearly a "salt and pepper" pattern of expression along the gut line in a region that likely represents the zebrafish kidney (Figure12). Transcripts for *pacap1b* has been detected at the level of telencephalon, the diencephalon, the rhombencephalon and in neurons of dorsal part of the spinal cord (Alexandre and al. 2011). In our treated larvae we found a stronger coloration at the level of the spinal cord and, like for *vip* expression, in a "salt and pepper" fashion in the kidney (Figure12). bdnf is expressed at the level of the brain specifically in the cranial ganglion, the telencephalon, the peripheral olfactory organ, the neuromast (Thisse et al. 2004); after the thermal stimulus it showed expression in the same regions but at stronger levels (Figure13).

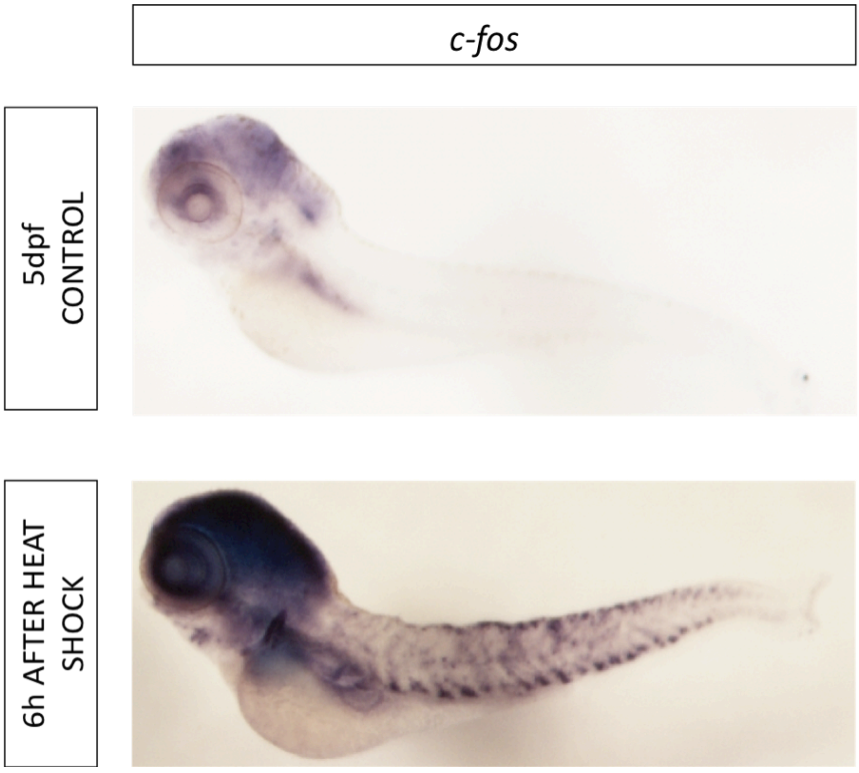

Figure10

Figure10: *c-fos* over-expression at 6h after the heat shock in contraposition with the physiological *c-fos* expression in a 5dpf control embryo. The heat shocked embryo presents an over-expression at the level of the spinal cord and also in the brain, where usually is expressed.

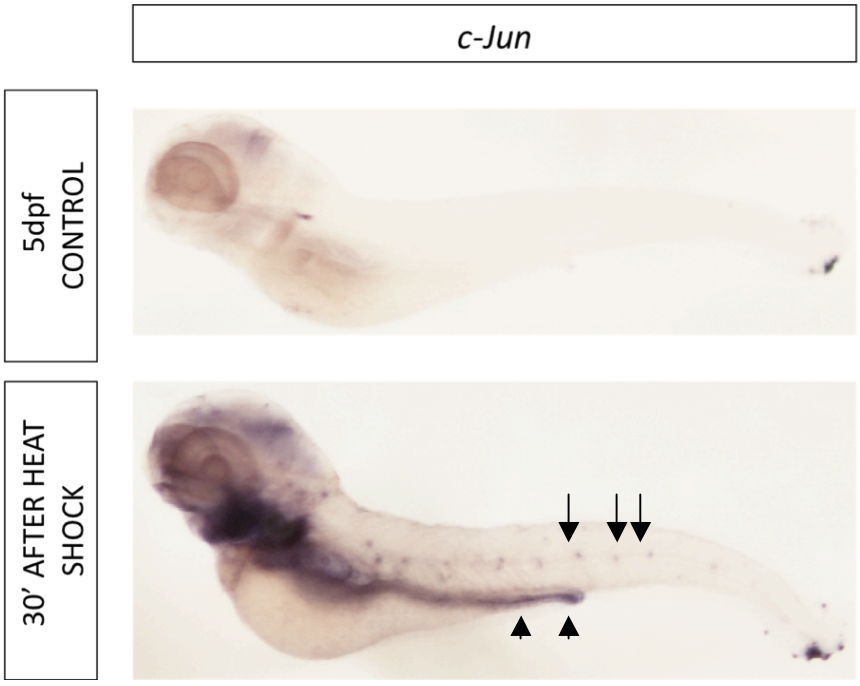

Figure11

Figure11: 5dpf *c-jun* control shows a different staining from the experimental sample, over-expression in the periphery of the gut region (arrowheads) with a diffuse coloration after 30'.
We notice also spots, along the body, that may be neurons of the lateral line (arrows).

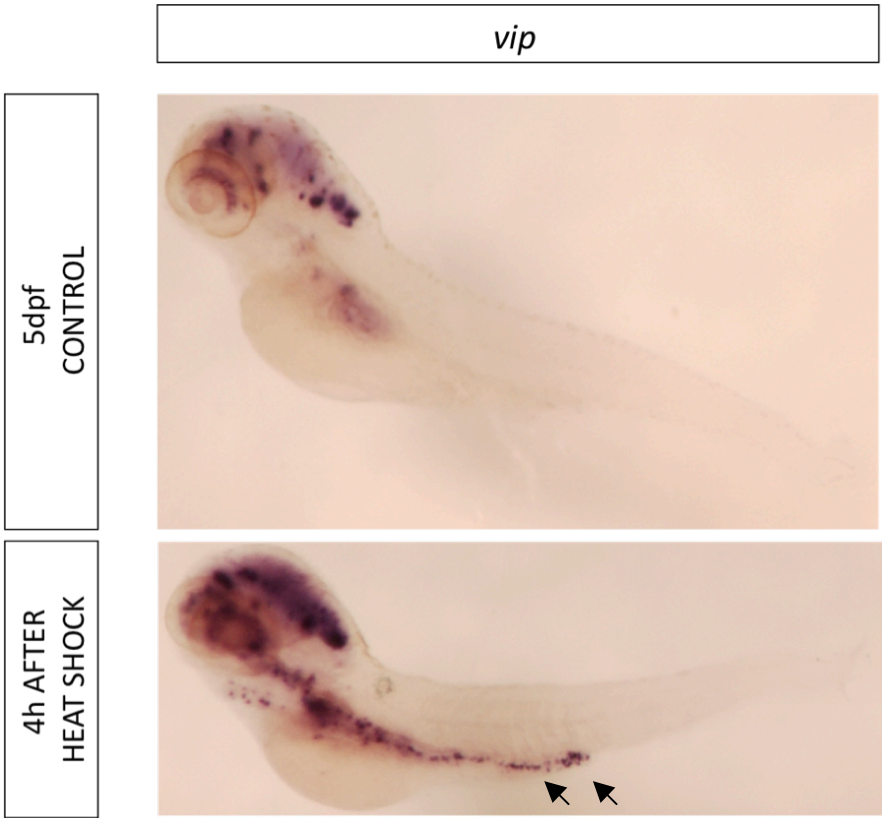

Figure12

Figure12: *vip* over-expression in a “salt and pepper” coloration along the gut region which are the kidneys (arrows), at 4h after the thermal stimulus in contrast to the 5dpf control staining.

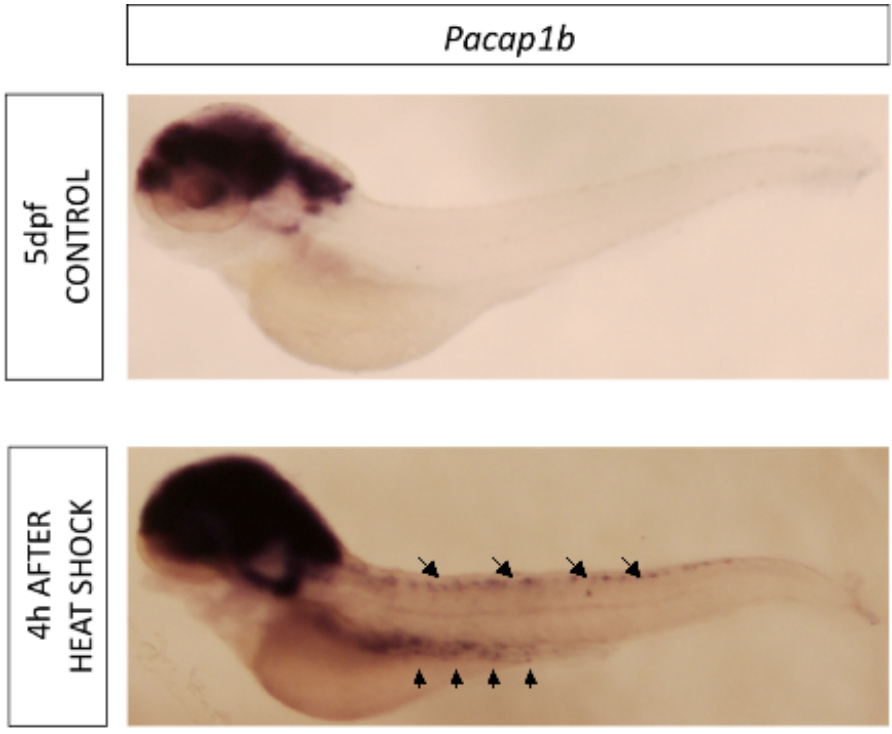

Figure13

Figure13: fish fixed after 4h from the heat shock test in which *pacap1b* appears to be over-expressed at the level of the kidney (arrowheads) and in the spinal cord (arrows) in contrast to the control coloration

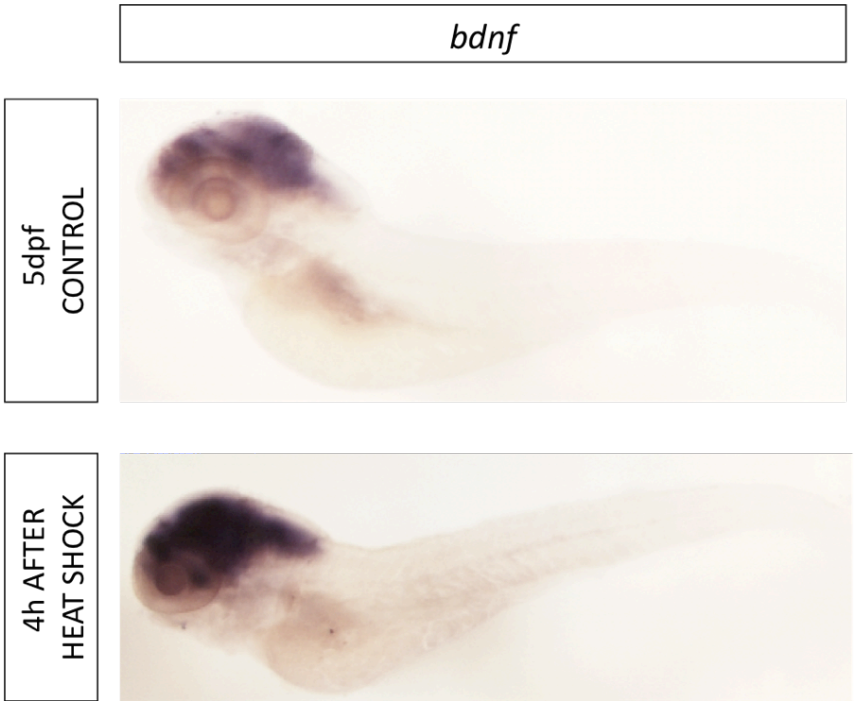

Figure14

Figure14: *bdnf* at the 4h time point after the nociceptive stimulus is over-expressed only in the same brain regions where its is normally present.

We performed hand-made transversal sections of representative heat treated larvae to confirm the over-expressed gene localization. We found *c-fos* staining in single cells within the spinal cord and *c-jun* within the lateral lamina and the gut (Figure15). We confirmed that the coloration for *vip* and *pacap1b* is outside the gut in the kidney (Figure16).

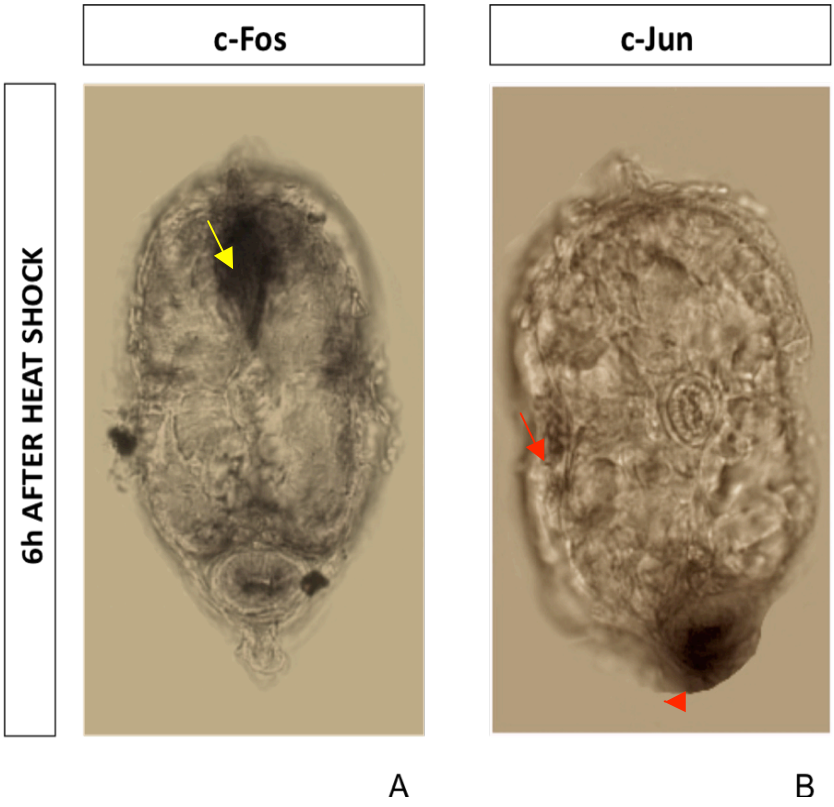

Figure15

Figure15: transverse trunk section of 2 embryos fixed at 6h (A) and 30'(B) after the heat shock and hybridized with *c-fos* probe (A) and *c-jun* probe (B). Yellow harrow indicates the area of the spinal cord with cells positive to *c-fos*; red arrow indicates cells of the lateral line and black arrowhead indicates cells around the gut all positive for c-jun.

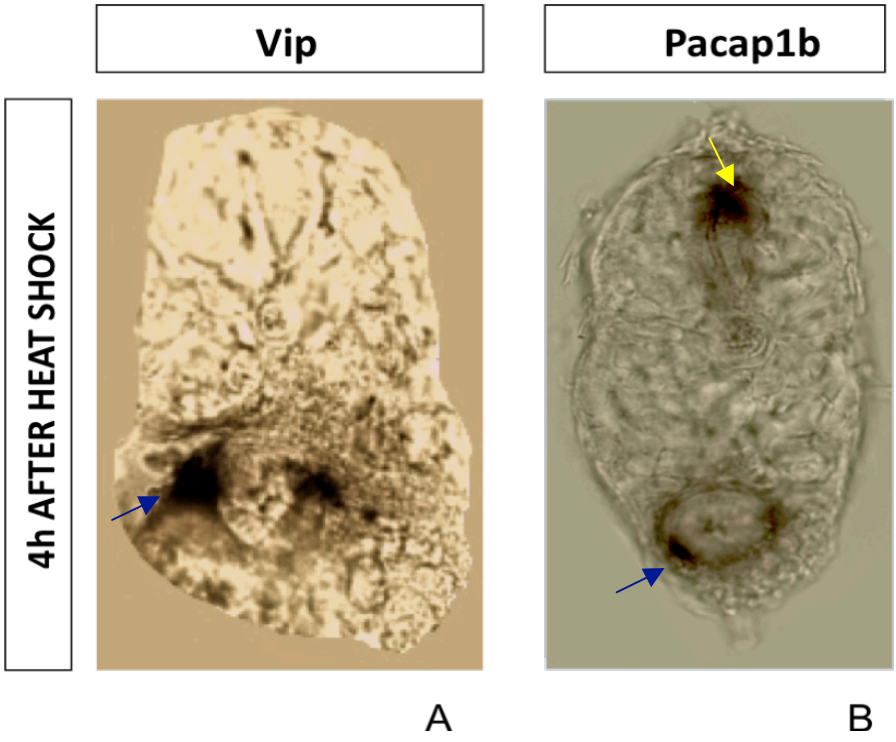

Figure16

Figure16A: transverse trunk section of a 4h post heat shock larvae hybridized with *vip* probe: here we can see the coloration at the level of the kidney (blue arrow).
Figure16B: transverse trunk section of a 4h post heat shock larvae hybridized with *pacap1b* probe: here we can see the coloration at the level of the kidney (blue arrows) and in the spinal cord (yellow arrow).

In the following table we reported the localization of the PMGs after heat shock in the spinal cord and the later line , which likeky are involved in (Table6).

Gene	Localization	5dpf ctrl	30'	2h	4h	6h	24h
c-fos	Lateral line	0/14	14/14	10/11	2/8	7/14	1/12
	Spinal cord	0/14	0/14	1/11	2/8	5/14	1/12
c-jun	Lateral line	0/12	7/10	0/14	0/14	0/14	0/14
	Spinal cord	0/12	0/10	0/14	0/14	0/14	0/14
pacap1b	Lateral line	0/12	1/14	0/13	0/12	0/13	0/14
	Spinal cord	0/12	0/14	7/13	7/12	11/13	3/14

Table6

Table6 : *c-fos, c-jun, pacap1b* over-expression in the spinal cord and the lateral line in larvae after heat shock experiments.

Trpv1 cloning

Zebrafish larvae can behaviorally respond to excessive heat or cold (Prober et al 2009) and upon noxious heat stimulus show a dynamic activation of the PMGs in space and time, suggesting that 5dpf zebrafish larvae have molecular receptor for sensing noxious heat. Some of these PMGs show ectopic expression in what we think are the neurons of the lateral line, which is a sensory tissue specific of the fish. Quite striking, at *c-fos* and *pacap1b* show over-expression in neurons of the spinal cord as we postulated in our original hypothesis. The best candidates receptors for sensing noxious stimuli are the transient receptor potential (TRP) family. Among this family the TRPV subtypes are the receptors that mediate noxious heat in mammals and TRPV1 is the most used for this function. Considering that most of the PMGs are early responsive genes, their activation after noxious heat could be also explained as a cell stress response. Taking advantage of this reason, cloning the receptor for noxious heat would be instrumental to determine if any of the PMGs activation is a real response to thermal noxious stimuli.

Thus we cloned zebrafish *trpv1* cDNA to determine its role in noxious thermal sensing. We prepared cDNA from 5dpf larvae and cloned the gene in 3 fragments (A-B-C). We designed 3 couples of primers in order to have ~1000bp for each fragments (Table 1). Then, we joined A-B-C fragments with a PCR based methodology in order to obtain the full length cDNA.

trpv1 cDNA sequence presents one nucleotide variation respect the annotated sequence (GenBank: EU423314.1) that leads to a different codon. In fact, our sequence showed an arginine instead of a glutamic acid in position 1167bp starting from the ATG. For this reason we analyzed the receptor domains (Smart Mode, Letunic et al 2008) and we found out that this mismatch takes place in a not significant domain for the receptor activity. Moreover, we compared our sequence to that one from the Homo sapiens and the mouse finding out, in that domain, the same aminoacid of our sequence. Therefore, we concluded that the cloned cDNA presents a polymorphyism of zebrafish *trpv1* (Figure18).

We subcloned TRPV1 in pCS2-Flag expression vector using a PCR approach: we designed a set of primers (Table 1) including specific restriction enzyme sites present in pCS2-Flag in order to cut and clone the cDNA in frame with the pCS2-Flag. In this way we could use anti-Flag monoclonal antibodies to confirm the cloning and localize the cells where TRPV1 is expressed.

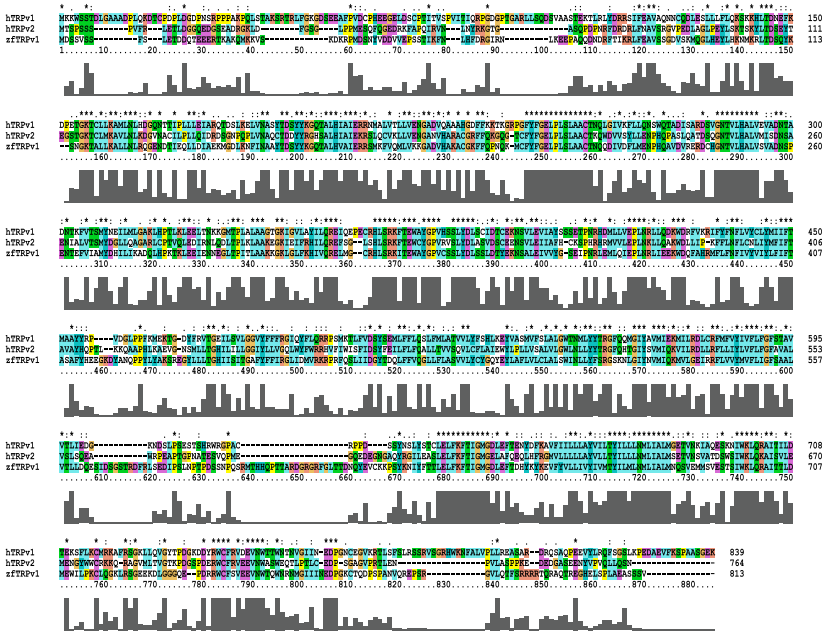

Figure17A

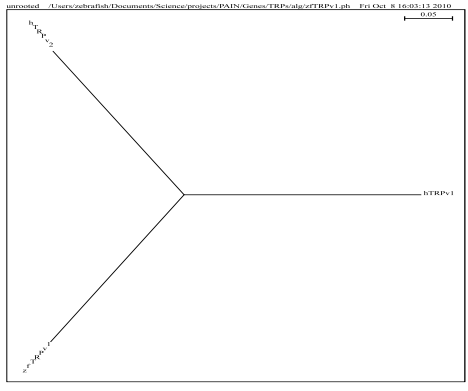

Figure17B

Figure17 : (A) alignment of human *trpv1*, human *trpv2* and zebrafish *trpv1* sequences using ClustalX program. (B) phylogenetic tree made with Njplot .

TRPV1 expression pattern in 5dpf zebrafish larvae

In this essay we wanted to verify if *trpv1* was expressed in the regions of the larvae that are known to contain sensory neurons. Thus, we performed an in situ hybridization experiment using *trpv1* probe in 5dpf larvae. We found *trpv1* expressed at the level of the gut, the trigeminal ganglia and the brain (Figure19). We also know that trpv1 is expressed in the spinal cord at early stages, we did not see this expression in 5dpf larvae, but this can not exclude that TRPV1 proteins are present in spinal cord neurons and/ or that *trpv1* is expressed at very low level in these cells.

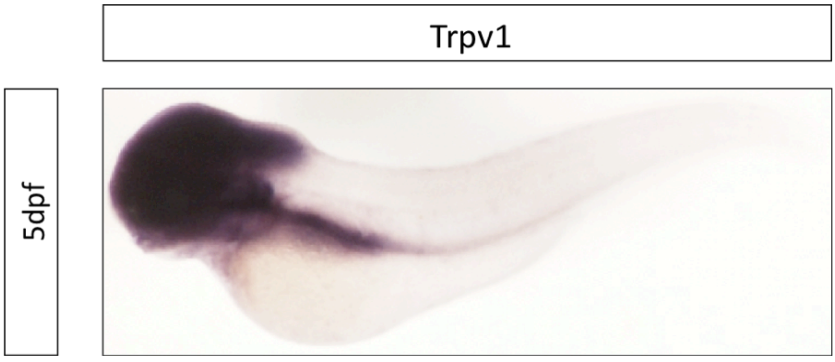

Figure18

Figure18: *trpv1* expression pattern at the level of the gut and the brain, including the trigeminal ganglia.

Zebrafish *trpv1* is properly expressed and localized in HEK 293T cell lines

We sub-cloned *trpv1* in pCS2-Flag in order to express this cDNA in human cells to test functionally *trpv1* ability to respond to various stimuli. To test if Trpv1 is correctly expressed, translated and localizated in Hek293T cells we assayed , by immunohistochemistry, cells transfected with pCS2-*Flag-trpv1* and with an empty vector like control. We used an anti-Flag antibody as a primary antibody and a goat anti mouse-FITC like secondary antibody, while the cells nuclei were stained with DAPI. Samples were visualized in a fluorescence microscope (Figure19). In cells transfected with pCS2-*Flag-trpv1* we detected a clear Flag-Trpv1 expression in the cytoplasm but stronger at the level of the cellular membrane, while the Flag alone showed a more homogeneous expression.

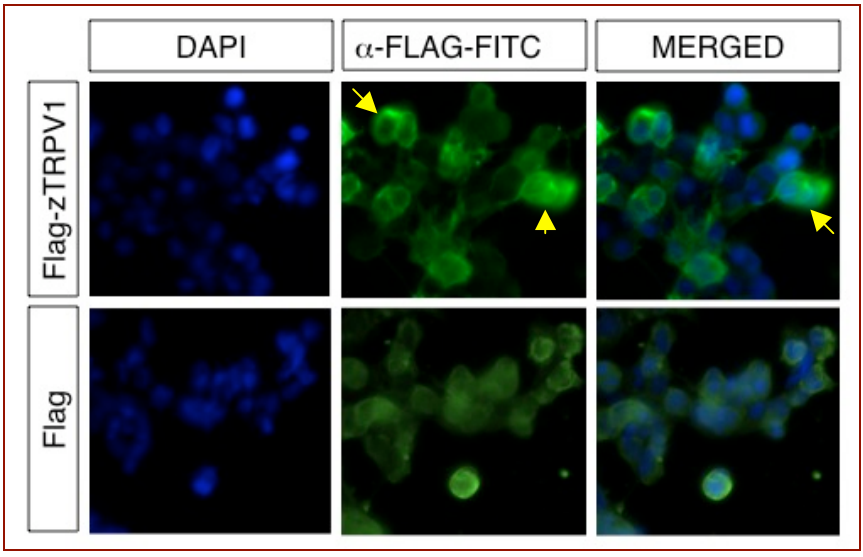

Figure19

Figure19: TRPV1 is expressed at the level of the cellular membrane, yellow arrows. Flag alone is homogenously distributed in the cells.

Zebrafish TRPV1 senses protons but not chemical or noxious stimuli

With our collaborator Dr. Bruce Bryant of the Monell Institute of Philadelphia we tested the ability of TRPV1 to sense chemical or thermal noxious stimuli analyzing changes in intracellular calcium levels, [Ca^{2+}]. These changes were measured using ratiometric digital fluorescence calcium imaging (Grynkiewicz et al., 1985). Graphic in fig 20A shows the average response of >150 regions of interest (cells) in cells transfected with pCS2-*Flag-trpv1*. The common irritant present in hot pepper, *capsaicin*, did not stimulated any response in the experimental cells. Heat at 48^{o}C produced a sharp increase of fluorescence that is not determined by the TRPV1, but is a known artifact due to the effect of heat on the fluorescent dye. In fact there is an equivalent increase in the control sample (fig 20B). When we checked response to protons, only the cells transfected with pCS2-*Flag-trpv1*, not the control, showed a strong activation. This response was replicated twice obtaining the same result. Finally, all the samples responded to carbachol, which is a well known muscarinic receptor agonist used in this test as positive control, showing that the cells were alive and active. Thus, zebrafish TRPV1 in an heterologous system is not able to sense noxious heat and capsaicin, while is responding to high proton concentration (pH 4.5).

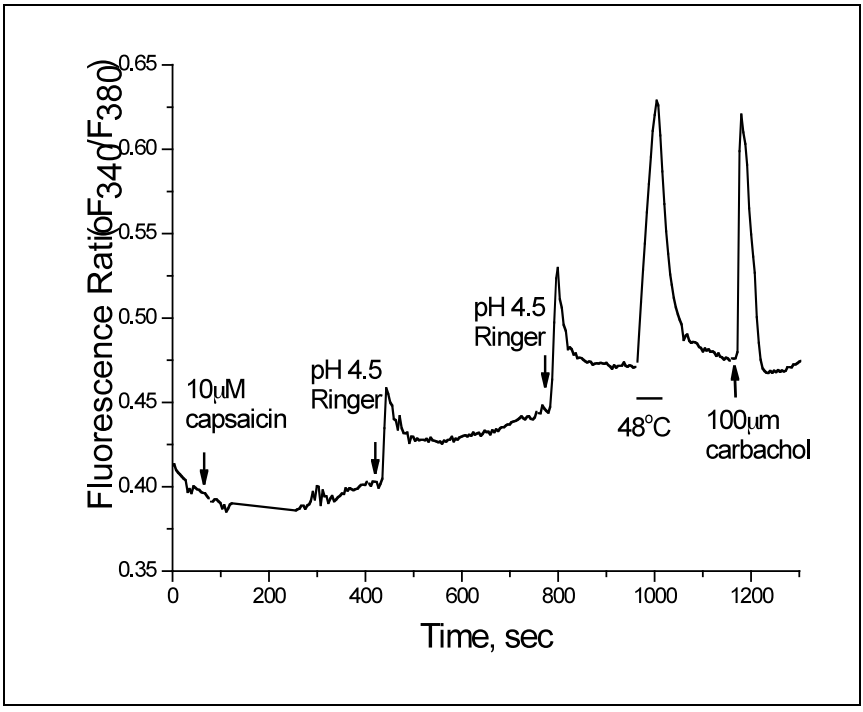

Figure20A

Figure 20A: HEK2093 cells transfected with zebrafish pCS2-Flag-TRPV1.

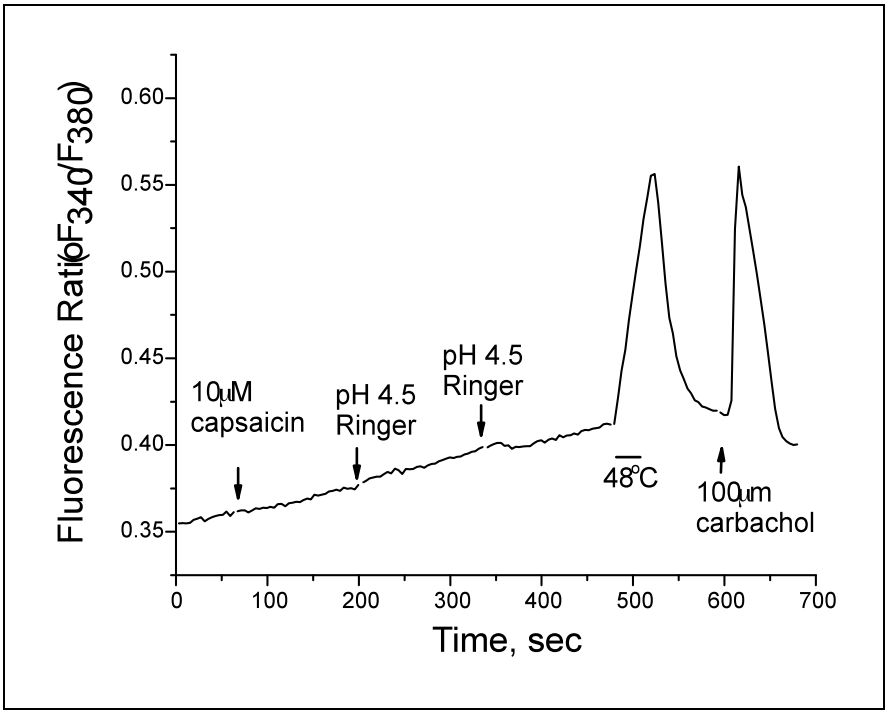

Figure20B

Figure20B: HEK2093 cells transfected with pCS2-Flag.

Knock-down of zebrafish trpv1

To investigate the functional role of Trpv1 receptor in the mediation of the thermal stimulus, we used a *trpv1* morpholino oligomer against the ATG of trpv1 in order to stop mRNA translation and eventually block the thermal nociceptive pathway that lead to the PGMs over-expression. We injected 1-2pL/embryo of 0.5mM, 1mM and 2mM antisense morpholino in 2-4 cells embryos to test the highest concentration of morpholino that can be used in our experiments without kill the embryos. We found that the morpholino injections were nicely tolerated by the embryos, so that we could use the 2mM concentration. Thus, we verified *trpv1* mRNA level after 3, 4 and 5dpf by RT-PCR. Our data show that Trpv1 morpholino worked at 3 and 4 days from the injection, blocking the mRNA expression (Figure21). However, at 5 days trpv1 mRNA expression was restored: we supposed the morpholino concentration is too diluted in the cells of a 5dpf embryo to have a gene expression blocking function.
We did not see evident phenotype after *trpv1* morpholino injection, however, we need to look more carefully to the development of the trigeminal ganglia and the spinal cord neurons. In our laboratory we are now growing a transgenic line that expresses GFP under control of the *ngn1* full enhancer/promoter that will allow us to visualize the trigeminal ganglia and spinal cord neurons *in vivo*. We are planning to inject with *trpv1* morpholino embryos of Tg (*ngn1:egfp)* line to determine the effect of the TRPV1 in zebrafish trigeminal ganglia and spinal cord neurons development.

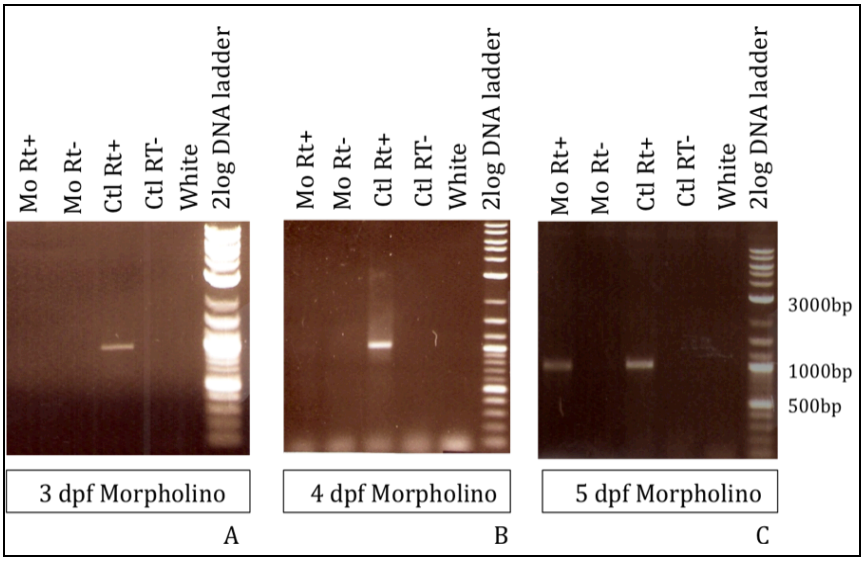

Figure21

Figure21: in 3dpf and 4dpf embryos morpholino works blocking the expression of t*rpv1* mRNA. The only band in the picture shows the *trpv1* mRNA expression in the 3dpf and 4dpf control (A-B). In 5dpf embryos the morpholino doesn't block *trpv1* mRNA expression so we see the expected RT-PCR product MoRt+ . Primers used in Table1. Beginning from the left side of each pictures we loaded: morpholino Rt+, morpholino Rt-, Rt+ from control cDNA, Rt- from control cDNA, white sample and the 2log DNA ladder (Neb Biolab).

DISCUSSION

In this study we showed a new way to use *Danio rerio* larvae in nociception studies. We are convinced that zebrafish would be an exceptional animal model for this type of studies. We established the methodology for an easy thermal nociceptive essay in which 5dpf embryos were dip in water at 48°C for 5 seconds.This assay results into the over-expression of *c-fos*, *c-jun*, *vip*, *pacap1b* and *bdnf* (pain markers genes, PMGs). In-situ hybridization experiments showed peculiar over-expression of the PGMs in time and space. *c-fos* and *c-jun* are known to be early stimulus responsive genes and in our data they showed the same behavior, with an over-expression right in 30' after the heat shock. *vip*, *pacap1b* and *bdnf* presented a response to the thermal stimulus with the higher over-expression around the 4h after the test. The in situ analysis helped us to discriminate between two waves of activation: an early activation within 30min from the stimulus when the PMGs were over-expressed in many tissues, and a late wave of over-expression when *c-fos*, *c-jun* and *pacap1b* were expressed in single cells of the spinal cord or in the sensory lateral line neurons. This late wave of ectopic expression suggests that some pain marker genes are also induced after painful stimuli in the zebrafish. Therefore the mechanism linking noxious stimuli and the expression of PMGs is largely unknown, it can be considered evolutionary conserved.

In addition, in *vip and pacap1b* we found consistently conserved an over-expression in the kidney. Quite interesting another member of the TrpV family, *trpv4*, is expressed in the pronephric duct of the developing larvae (Steve Mangos, 2007), thus TRPV4 cold be the heat receptor in this cell types. To confirm the PMGs localization we performed transverse hand-made sections, however, new and more precise analysis, using additional markers of each cell type will be necessary to confirm our results.

On the attempt to understand if these results are linked to nociception and they are not just cellular stress consequences, we cloned and study TRPV1 functions. We showed, with in situ hybridization, that *trpv1* mRNA is expressed in structures of the 5dpf larvae that are relevant for nociception, like the trigeminal ganglia. We set up loss of function experiment using *trpv1* morpholino in order to block the expression of this receptor and see if, in his absence, we have or not

the same response to the thermal stimulus. However, we determined that the morpholino we used works at 3 and 4dpf but not at 5dpf so we could not repeat our noxious heat test. In addition our functional studies in human HEK293T cell have shown that zebrafish TRPV1 is only responsive to pH of 4.5 but not to capsaicin or heat.
However, it is well known that TRPV1 works in collaboration with other co-factors (Fernandes 2011) so we can assume that, to study its functional expression, we will need to look for TRPV1 integration factors. Thus, we still need to find a heat receptor in the zebrafish. Our next candidate for functional studies in cell lines will be TRPV4.
We now are preparing another strategy to finally link heat noxious stimuli and PMGs over-expression, we will perform our noxious heat test in embryos treated with tricane, an anesthetic that prevents action potential and neurons signaling. Our working hypothesis is that, stopping action potential, we could stop the signal of the peripheral nociceptors that sense heat to the spinal cord thus, we should not see ectopic activation of *c-fos* and *pacap1b*.
Considering the fuctional results with zebrafish TRPV1, we will determine a methodology to expose zebrafish larvae to low pH and check for PMGs expression.

CONCLUSIONS

In this work we have establish the methodology for an easy thermal nociceptive essay and showed that thermal noxious stimuli induce PMGs expression as well as in mammals. We have further cloned and functionally characterized *trpv1* receptor. However, we still need to link in a mechanical way the noxious stimulus with the activation of the PMGs. For this reason we will direct our future studies also on the characterization of other zebrafish TRP channels (TRPV4) and in preparing new methodology to test nociception. In this prospective our goal should be to demonstrate that different kind of noxious tests (thermal, mechanical, chemical) could be used in the fish to study nociception via simple *in situ* hybridization.

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Professional Career :
Since March 2009 Ph.D student in Cellular and Molecular Oncopatology, Faculty of Medicine and Surgery, University of Palermo.

Professional Societies Membership:
-2011 Mid-Atlantic SDB Meeting 3-5 June 2011, University of Pennsylvania, Philadelphia, PA.
-October 2011 : member of the Noldus Zebrafish Research Panel.

Scientific Activities and Oral Presentations:
-Temple Developmental Biology Joint Lab Meetings, Evo-Devo: 19/01/2011- 20/04/2011
-Temple Developmental Biology Joint Lab Meetings, Neurolunch: 12/02/2010- 19/11/2010
-Temple Developmental Biology Joint Lab Meetings, Zebrafish community: monthly meeting with oral presentations.

Activity of reviewer for papers:

-Buonocore et al, allodynic skin in post-herpetic, Journal of Cellular Physiology 2011.

- Gene Mutation Effect of Aqueous and Methanol Extracts of Salted Fish from Pulau Pinang, Malaysia towards V79 Lung Fibroblast Cells, JZUS-B (Biomedicine & Biotechnology) 2011.

BOOKS, PAPERS AND ABSTRACTS PUBLISHED DURING THE PHD COURSE

-Puca, Malafoglia, Giordano, Cancer Stem Cells: Targets for Future Cancer Therapies. October 11, 2011; Book- Stem Cells: From Mechanisms to Technologies

-Marfe et al, Blood derived stem cells: An ameliorative therapy in veterinary ophthalmology.
J Cell Physiol. 2011 Jul 25.