

5-30-2008

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Siobhan C. Reilly
University of Liverpool

John P. Quinn
University of Liverpool

Andrew R. Cossins
University of Liverpool

Lynne U. Sneddon
University of Liverpool

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Recommended Citation

Reilly, S. C., Quinn, J. P., Cossins, A. R., & Sneddon, L. U. (2008). Novel candidate genes identified in the brain during nociception in common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). *Neuroscience Letters*, 437(2), 135-138.

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Novel Candidate Genes Identified in the Brain during Nociception in Common Carp (*Cyprinus carpio*) and Rainbow Trout (*Oncorhynchus mykiss*)

Siobhan C. Reilly, John P. Quinn, Andrew R. Cossins, Lynne U. Sneddon
University of Liverpool

KEYWORDS

CNS, microarray, nociception, Teleost fish, Vangl2

ABSTRACT

Recent studies have demonstrated that teleost fish possess nociceptors that detect potentially painful stimuli and that the physiological properties of these fibres are markedly similar to those found in mammals. This finding led to suggestions of possible pain perception in fish, contrary to the view that the sensory response in these animals is limited to the spinal cord and hindbrain and as such is reflexive. Therefore, the aim of this study was to determine if the brain is active at the molecular level by using a microarray analysis of gene expression in the forebrain, midbrain and hindbrain of two fish species. A comparison between the two species at different time points showed that many genes were differentially regulated in response to a noxious stimulus compared with controls. A number of genes that are involved in mammalian nociception, such as brain-derived neurotrophic factor (BDNF) and the cannabinoid CB1 receptor were regulated in the fish brain after a nociceptive event. Novel candidates that showed significant regulation in both species were also identified. In particular, the Van Gogh-like 2 gene, was regulated in both carp and trout and should be pursued to establish its precise role in nociception.

The advantage of using a global transcriptomic approach to study nociception is that the expression of thousands of genes can be monitored simultaneously [13], which confers the ability to identify novel candidate genes involved in nociceptive transmission and modulation. Identifying the genes that are regulated after nociception may lead to novel potential targets for pharmacological alleviation of pain.

To date, all of the microarray experiments focusing on pain processing have examined mammalian DRG or the spinal cord [3,8,16,25,28]. These studies have identified several hundred candidate genes for involvement in pain transmission but most are yet to be validated. Transcriptional regulation in the brain is also relevant because it is a vital anatomical region for processes involving the affective component of pain [12]. Therefore, it is important to investigate which genes are regulated in the brain after a nociceptive stimulus, as genes may be identified that were not detected in microarray studies of DRG and spinal cord.

Fish possess nociceptors with electrophysiological properties identical to those of mammalian nociceptors [20–22], but the issue of pain perception is undecided. Questions regarding the capacity for pain in fish have centred on whether the brain is active during noxious stimulation or whether it is limited to a reflex

response at the level of the spinal cord or hindbrain [17]. The objective of this study was to conduct a high-throughput analysis of gene expression in common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) to demonstrate that genes are regulated in the fish brain after a nociceptive stimulus and therefore show that the brain is active at the molecular level. The study aimed to identify genes that are differentially expressed in response to nociceptive treatment in the two fish species. The resulting gene lists can be compared with those from mammals to indicate genes that are evolutionarily conserved in nociception and may also reveal new candidates.

Experimentation was conducted in an ethical and humane way under approval from Liverpool University's Local Ethical Committee and under Home Office (UK) licensing. Common carp (*C. carpio*, control and treatment: specimens were used for each time point) were obtained from a commercial supplier and held in a stock tank (2m x 2m x 0.5m) for 3 months. Experimental fish were randomly chosen from the stock tank and moved to individual tanks (50cm x 30cm x 30 cm) which were maintained at ambient temperature (18 °C). Tanks were filled with de-chlorinated freshwater which was continuously aerated via an air stone connected to airline in a closed filtered water system (Interpet Series 1, UK), where one-third of the water was exchanged for fresh water every 2 days and had a 12 h:12 h light–dark regime. They were left for 1 week to recover from possible handling stress and acclimate to the new surroundings. During this period, the fish were fed with 1% body weight of red mosquito larvae per day.

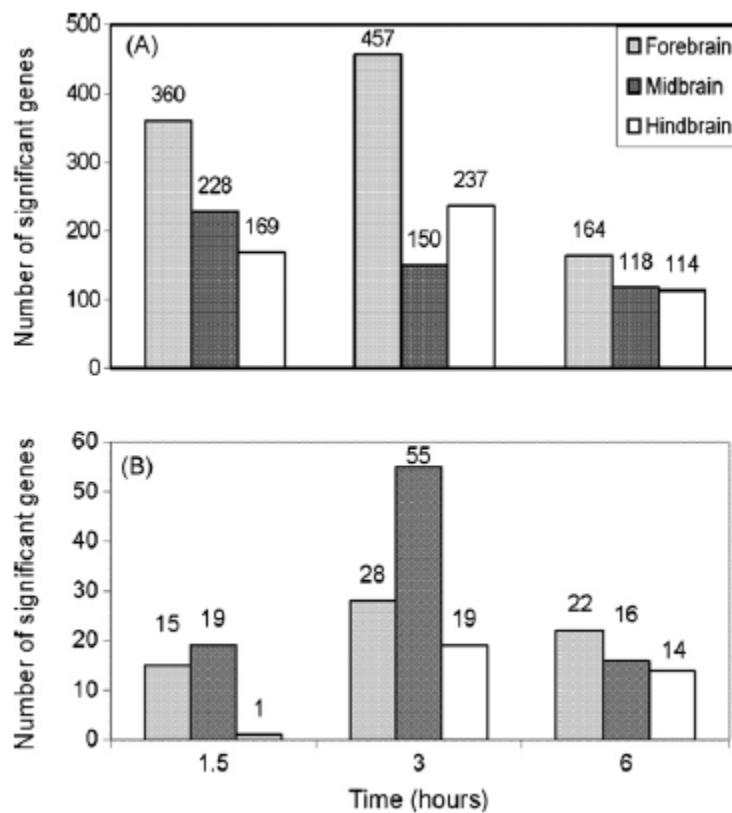
Rainbow trout (*O. mykiss*, control and treatment: specimens were used for each time point) were obtained from a commercial supplier. Fish were maintained in a stock tank for 3 months at 10 °C with a 12 h:12 h light–dark regime in an open water system and fed daily with 1% body weight. Trout were then moved to individual tanks (35cm x 30cm x 30 cm) for 1 week. All tanks were screened from visual disturbance and observations were made through small openings in the screening.

Fish were individually anaesthetised in a 25-L bucket containing 10 L of aerated water dosed with benzocaine (10mL of 1 g/30mL ethanol). Once deep plane anaesthesia was achieved, sterile physiological saline (0.9% NaCl; 0.1mL) was injected (1mL syringe, 25 g needle) subcutaneously into the upper and lower frontal lips of the fish belonging to the control group and the same volume of acetic acid (5% acid, diluted in saline) was injected into the treatment groups. The total volume of liquid injected into each animal was 0.1mL. This paradigm has previously been validated as a model of nociception as nociceptors are activated [21] and an adverse behavioural response is observed [20]. Fish were returned to their original tanks and killed by concussion followed by severing the spinal cord at 1.5, 3 and 6 h after treatment. The brain was divided into forebrain (olfactory lobes and telencephalon), midbrain (diencephalon and optic lobes) and hindbrain (medulla, pons, cerebellum and brain stem) and stored at –80 °C.

The protocol consisted of a reference-based array design where the experimental samples were compared to a common reference. A dye swap was incorporated into the design to account for dye bias; in the forward reaction, Cy3 was used to label the reference sample and Cy5 was used in the experimental sample and *vice versa*. In total, the array design consisted of 72 arrays for each of the two species, which comprised of arrays for both control and treatment specimens for each of the three time points, in each of the three brain regions. In addition, each array had a dye swap replicate as well as a technical replicate. The carp array is described fully by Williams et al. [26] as carpARRAY version 1 and consists of 13,440 probes. To make a direct comparison of gene expression between the two species, RNA extracted from rainbow trout was also hybridised to the common carp array. Information about the probe sequences and length can be accessed at the carpBASE website [23]. The carp array in this study is fully annotated and available in the public database ArrayExpress [26] and the raw data files are currently under submission to this database.

Tissue was pooled for each brain region and mRNA was extracted using TRIzol reagent (1 mL/100mg tissue). Reverse transcription was conducted using Stratascript II (Stratagene; Cambridge, UK). Samples were labelled with 2.5mM Cy dye (Amersham, Buckinghamshire, UK). The target (labelled cDNA) was denatured by heating for 2 min, and then applied under 22mm x 60mm lifter slips (Implen; Munich, Germany) to the microarray slides. Slides were incubated in a hybridisation chamber (Genetix, New Milton, UK) at 65 °C for 17 h, after which they were washed with wash buffer I (1x SSC, 0.1% SDS) and wash buffer II (0.1 x SSC). Arrays were scanned with a Genepix 4000A laser scanning microscope (Molecular Dynamics; Sunnyvale, CA, USA) and data were analysed with GeneSpring statistical software (Agilent, Santa Clara, CA, USA).

Fig. 1. Fold-change values for treatment compared to control expression values in common carp (A) and rainbow trout (B). Normalised expression values in control and treatment were compared with a one-way ANOVA for each time point and brain region. The number of genes regulated ($P < 0.05$) in each of the groups is plotted.



Several normalisation and data transformation methods were used in the following order. The first data transformation step accounted for the dye swap, in which reference and experimental samples were labelled with alternating Cy dyes, and this accounted for fluorophore-derived variation in intensity. Second, \log_2 expression intensity measurements of less than 0.01 were set to 0.01. Finally, a Lowess [29] transformation was used to account for dye-specific intensity-dependent non-linear effects using 40% of the data for smoothing. Unreliable data points were removed by using only genes which possessed a detectable signal in at least 50% of the arrays being analysed. Genes with statistically significant differences in treatment and control were identified with a one-way ANOVA test. The ANOVA test incorporated Welch's T -test, which does not assume equal variance. To produce lists of significantly

changing genes for each species, control expression values were compared to treatment samples separately in each time point and brain region. At this stage a false discovery rate was not used, as it was likely to create false negatives and any false positives could be discounted at a later stage in the analysis.

The accuracy of the array data for several genes was validated by real-time RT-PCR. The QuantiTect SYBR Green (Qiagen, Crawley, UK) protocol was used with a RotorGene RG-3000 (Corbett research, Cambridge, UK) LightCycler system. The real-time RT-PCR was conducted in triplicate and the mean was used in later calculations. Gene expression was normalised to GAPDH and the differential expression ratio was calculated using the Pfaffl equation [11]. The primers used were: CB1 (5'-GGAGAGGAGTAACATGAGCT-3' and 5'-CAAGCCACCCAACCTTCTTGT-3') *Vangl2* (5'-CGGTCCCTTTGGTGACTCTA-3' and 5'-CCTTGGATGTGGTAGCCGTT-3'), claudin g (5'-CAAAGCACAGCTATGACCATG-3' and 5'-GATCTGTTCGCCCTCGCATC-3') and GAPDH (5'-TGATCGTTGATGGTCAGGC-3' and 5'-ATCACAAACATGGGTGCGTC-3'). Northern blotting and *in situ* hybridisation may provide more detailed analysis of changes in gene expression for the most promising candidate genes.

Genes were significantly regulated in carp, with most activity occurring in the forebrain at all-time points (Fig. 1A), indicating the viability of higher processing during nociception. The time points which displayed most regulated genes in carp were 1.5 and 3 h. There were fewer genes regulated in the trout (Fig. 1B), possibly because the trout cDNA was hybridised to a carp microarray, which may have distorted the data set [4]. However, Renn et al. [15] found that species with a divergence time of up to 200MYA could be used in heterologous hybridisation cDNA microarray studies in fish. The peak of gene regulation in trout occurred at 3 h and the forebrain and midbrain were the regions in which most gene regulation was detected. Similarly to carp, by 6 h gene regulation in trout had also diminished, and this reflects behavioural observations, where most anomalous behaviours are observed during earlier time points [14].

A number of genes were regulated in the carp brain at several time points throughout the experiment. As the multiple time points for each brain region were collected from different fish, this biological independence gave extra statistical weight to the inferences drawn. The genes which featured most commonly and in biologically independent lists in the carp are displayed in Table 1. The *Vangl2* gene was significantly regulated at 1.5, 3 and 6 h in the carp forebrain in noxiously stimulated fish. Additionally, *Vangl2* was also up-regulated in the trout forebrain, midbrain and hindbrain. In the carp brain, claudin g was regulated at various time points in different parts of the brain. Claudin g was also down-regulated in the trout hindbrain at 6 h.

In the carp, the calcium-binding protein, S-100 protein β chain, was down-regulated in the forebrain but up-regulated in the midbrain. Another calcium-binding protein, calmodulin 2δ , was differentially expressed after nociception in multiple time points and brain regions. The solute carrier family 25α gene was significantly regulated in all carp brain regions at 1.5 h and in the forebrain and midbrain at 3 h. The most dramatic change for this gene was in the hindbrain at 1.5 h, with a decrease of 5.22-fold. Expression levels of the transcription factor E4tf1 60 were found to be down-regulated in the forebrain. However, this transcript was upregulated in the midbrain and the hindbrain. Voltage-dependent anion-selective channel was down-regulated at 1.5 h in both the hindbrain and midbrain. It was also down-regulated at 3 h in the forebrain, yet it was up-regulated at this time point in the hindbrain as well as at 6 h in the midbrain.

Ependymin precursor (EPD), a neuronal cell adhesion molecule (NCAM), was up-regulated at 1.5 h in the carp midbrain, but a different response is seen in the hindbrain at this time where it is down-regulated. It was also down-regulated in the hindbrain at 6 h. Beside EPD, two other NCAMs were significantly regulated. These genes are of interest because they are involved in synaptic plasticity and learning and memory. Other NCAMs regulated include NCAM L1 precursor, which was up-regulated 1.92-fold in the

forebrain at 1.5 h and down-regulated 2.77-fold in the hindbrain at 3 h. A putative NCAM was up-regulated 1.44-fold in the forebrain at 6 h.

Several genes that are involved in mammalian nociception were significantly regulated after nociceptive treatment. A gene encoding the cannabinoid 1 (CB1) receptor, which is involved in mammalian nociception and analgesia [2], was up-regulated at 6 h in the carp forebrain and midbrain. The kainite receptor is activated by glutamate, which is an important neurotransmitter during nociception [18] and was down-regulated 1.43-fold in the carp forebrain at 1.5 h. Brain-derived neurotrophic factor (BDNF) is regulated during mammalian pain [9] and was also up-regulated 1.38-fold in the carp midbrain at 1.5 h. Synaptosomal-associated protein (SNAP) genes are regulated in mammalian dorsal root ganglion (DRG) during chronic pain [3,25,28], and in the present study, SNAP25B was down-regulated 1.86-fold in the trout forebrain at 6 h.

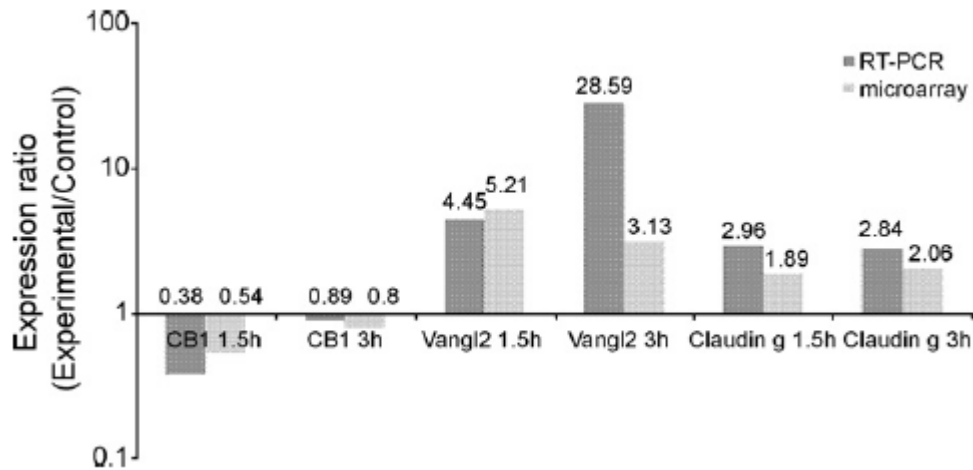
Table 1 Fold change of genes which were significant in multiple gene lists after microarray analysis of the carp brain

	Forebrain			Midbrain			Hindbrain		
	15h	3h	6h	15h	3h	6h	15h	3h	6h
Vangl2	5.21 ↑ (±0.46)	3.10 ↑ (±0.22)	1.93 ↓ (±0.06)						
Claudin g	1.92 ↑ (±0.06)	1.97 ↓ (±0.06)		1.89 ↑ (±0.14)	2.06 ↑ (±0.05)		1.94 ↑ (±0.05)	0.76 ↑ (±0.05)	1.53 ↓
S100 protein β chain	1.72 ↓		1.49 ↓	1.67 ↑	1.61 ↑ (±0.06)				
Solute carrier family 25α	1.65 ↑	1.73 ↓ (±0.12)		1.54 ↑	1.77 ↑		5.22 ↓		
E4tfl 60		1.80 ↓		2.22 ↑		1.69 ↑		2.14 ↑	
CB1	1.86 ↓		1.78 ↑			1.51 ↑			
SCD	1.50 ↑	1.76 ↑	1.24 ↑						
V-dependent anion-selective channel		1.48 ↓		1.34 ↓		1.51 ↑	3.03 ↓	2.19 ↑	
Ependymin precursor				1.32 ↑			1.81 ↓		1.42 ↓
Calmodulin 25		2.08 ↓ (±0.15)		1.53 ↑				1.80 ↑	

The fold change was the normalised intensity in treatment samples/normalised intensity in control samples. ↑ indicates up-regulation in treatment samples; ↓ indicates down-regulation in treatment samples.

A nociceptive experience was correlated with transcriptomic responses in the brains of both the common carp and the rainbow trout. Gene regulation was particularly evident in both the forebrain and midbrain in the three hours after the event. The molecular changes in these brain regions allow for the possibility that there are similarities in nociceptive processing between fish and higher vertebrates.

Fig. 2. The expression ratio (normalised expression in treatment samples/normalised expression in control samples) of CB1, *Vangl2* and claudin g in the carp forebrain was determined by real-time RT-PCR and is plotted on a log₂ scale. The ratio calculated by microarray analysis is also plotted. Expression of CB1 and *Vangl2* was measured in the forebrain and claudin g expression was measured in the midbrain. Expression was measured at both 1.5 and 3 h after a nociceptive or control stimulus.



The kainite glutamate receptor [27], the CB1 receptor [2] and BDNF [5] are genes that are involved in mammalian nociception. These genes were also regulated in carp, where they may also be modulating the nociceptive experience. In the current study mRNA encoding the kainite receptor was down-regulated in the carp forebrain at 1.5 h. This result was in agreement with Wang et al. [25], where concentrations of mRNA encoding the kainite receptor were reduced in the DRG after spinal nerve ligation, and it was suggested in this study that kainite receptor down-regulation removes inhibition of nociception and thereby causes hyperexcitability of nociceptive neurons. It is feasible that disinhibition is also occurring in the carp forebrain, resulting in hyperexcitability of neurones. In mammals, BDNF is up-regulated in the PAG after inflammation [5]. In the present study, BDNF was up-regulated in the carp midbrain at 1.5 h, and it is of interest to note that the homologue of the PAG in fish is situated in the midbrain. Expression values of three genes were validated by real-time RT-PCR and the results obtained were in agreement to the microarray results (Fig. 2).

Genes which were regulated in a microarray study of neuropathic pain in ratDRG[25], and also in the carp model of nociception include the S100 protein whereas the rainbow trout shared differential expression of SNAP25 genes with the rat [3,25,28]. Surprisingly, despite the evidence demonstrating a regulation in expression levels, further work is yet to be conducted into the role of SNAP25 genes in nociception. Genes identified in studies of rodent DRG or spinal cord, as well as in the brain of fish, such as SNAP25 and S100 protein, suggest not only a response across taxa, but also a system wide response.

Several genes with no known link to nociception were significantly regulated in both species and at multiple time points. The *Vangl2* gene has previously been implicated in development and is expressed heavily in mammalian cerebral cortex and DRG [24]. As a negative regulator of β -catenin-dependent transcription, *Vangl2* signalling can affect cell fate during development and also promotes c-Jun phosphorylation through the activation of the c-Jun N-terminal kinase (JNK) cascade induced gene expression [10]. In the current study, *Vangl2* was regulated in the carp forebrain, and in all regions of the trout brain. It is possible that *Vangl2* gene regulation may be co-ordinating other gene regulatory events.

The claudin-like protein claudin g (ZF-A9) was also regulated in both carp and trout. Claudin proteins are cell adhesion proteins which are a component of tight junctions [7]. Other members of the claudin family

have recently been shown to increase expression in the brain after inflammation [1], so the increased expression of these proteins could reflect the decreased integrity of the blood–brain barrier that occurs after inflammatory pain [6].

Several genes responded to noxious stimuli which are involved in neuronal cell adhesion and these genes are relevant to synaptic plasticity. One gene, a precursor for the cell adhesion protein ependymin (EPD), is intimately involved in learning and memory [19]. In the present study, EPD was up-regulated in the carp midbrain at 1.5 h. In contrast, it was down-regulated in the midbrain at 3 h and hindbrain at 1.5 h and 6 h. Neuronal cell adhesion molecule L1 was also up-regulated at 1.5 h in the carp forebrain, where it may be important in forming new memories. The involvement of NCAMs, particularly in the forebrain at 1.5 h, suggests that the resulting synaptic plasticity is part of the cellular response to noxious stimulation.

The current study has shown that gene regulation occurs in fish after a nociceptive event that is different to non-noxiously stimulated controls. Some of these genes are also regulated in mammalian nociception and, therefore, may have similar functional implications. This result indicates a level of conservation in the nociceptive response between fish and mammals. A number of genes may be considered as novel candidates for involvement in nociceptive processing since these are previously unlinked to nociception. Of particular interest is the *Vangl2* gene, as this gene was regulated in two species at three time points.

Acknowledgements

The work was funded by a BBSRC Ph.D. studentship grant. We are grateful to Gregor Govan for technical assistance and Margaret Hughes for printing arrays.

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