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Investigation of Van Gogh-like 2 mRNA Regulation and Localisation in Response to Nociception in the Brain of Adult Common Carp (*Cyprinus carpio*)

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KEYWORDS

Vangl2, fish, nociception, telencephalon (forebrain)

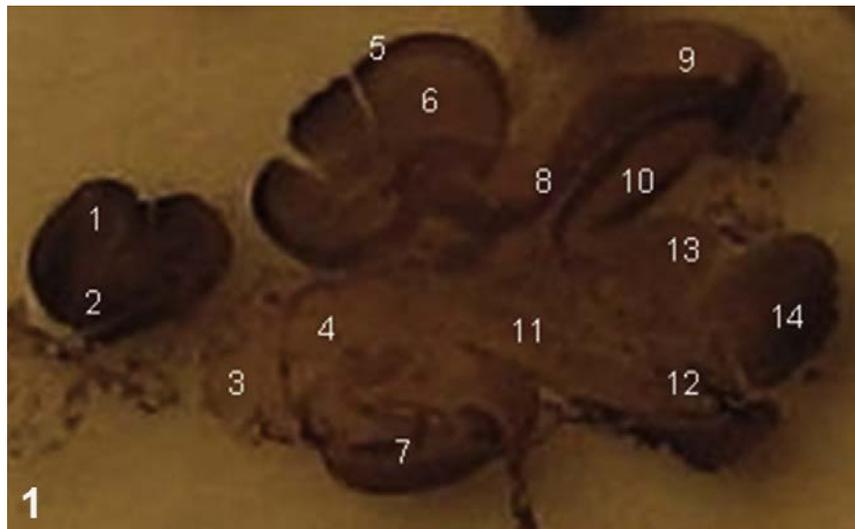
ABSTRACT

The Van Gogh-like 2 (*vangl2*) gene is typically associated with planar cell polarity pathways, which is essential for correct orientation of epithelial cells during development. The encoded protein of this gene is a transmembrane protein and is highly conserved through evolution. Van Gogh-like 2 was selected for further study on the basis of consistent regulation after a nociceptive stimulus in adult common carp and rainbow trout in a microarray study. An *in situ* hybridisation was conducted in the brain of mature common carp (*Cyprinus carpio*), 1.5 and 3 h after a nociceptive stimulus comprising of an acetic acid injection to the lips of the fish and compared with a saline-injected control. The *vangl2* gene was expressed in all brain regions, and particularly intensely in neurons of the telencephalon and in ependymal cells. In the cerebellum, a greater number ($P = 0.018$) of Purkinje cells expressed *vangl2* after nociception ($n=7$) compared with controls ($n = 5$). This regulation opens the possibility that *vangl2* is involved in nociceptive processing in the adult fish brain and may be a novel target for central nociception in vertebrates.

Van Gogh-like 2 (*vangl2*) is a member of the Van Gogh (Vang) or strabismus (Stbm) family of genes, which are associated with the planar cell polarity (PCP) pathway [23]. *Vangl2* has been reported in several types of cell, and is particularly well expressed in epithelial, mesodermal and mesenchymal cells, although it has also been implicated in the migration of cranial motor neurons in the hindbrain of fish [13]. In the developing mouse central nervous system, *vangl2* messenger RNA (mRNA) is preferentially expressed close to the ventricles, where it influences the closure of the neural tube [28]. After the neural tube has closed, other anatomical regions where *vangl2* mRNA is localised are: the cerebral cortex, dorsal root ganglia (DRG), olfactory epithelia, retina and the optic nerve [30]. Despite *vangl2*'s principal association with planar cell polarity (PCP) pathways, there is also potential for a post-natal involvement in sensory processing, due to the influences on transcription factors, as well as its intriguing location in DRG and the forebrain. Furthermore, the role of *vangl2* and Wnt signalling may have as yet unrecognised importance in the adult brain. Disruption of Wnt signalling has been proposed to cause neurological disorders [3], and have effects on synaptic plasticity in the hippocampus [2].

Nociception, the sensory mechanism for detecting potentially injurious stimuli that elicits pain sensation in humans, has been relatively recently explored in fish and is associated with molecular changes in the brain [25]. Since *vangl2* was shown to be up-regulated in fish brains after a nociceptive event in a microarray study [25], the aim of this study is to examine where *vangl2* mRNA is expressed in the brain of adult common carp, and whether transcript expression levels respond to noxious stimulation. The expression levels were assessed after 1.5 and 3 h with *in situ* hybridisation (ISH) using a specific riboprobe complementary to the *vangl2* transcript. Based upon previous research, it is hypothesized that mRNA encoding *vangl2* will be primarily localised to epithelial cells and the forebrain areas of the fish brain which are important in mammalian nociception.

Fig. 1. Common carp, sagittal section of the whole brain with RNA-ISH with *vangl2* riboprobe, Papanicolaou's haematoxylin counterstain. Expression of *vangl2* mRNA is seen ubiquitously throughout the brain. (1) Dorsal telencephalon; (2) ventral telencephalon; (3) optic chiasma; (4) thalamus; (5) tectum; (6) torus longitudinalis; (7) hypothalamus; (8) valvula cerebella; (9) corpus cerebella; (10) lobus caudalis; (11) tegmentum; (12) medulla oblongata; (13) cerebellar crest; (14) vagal lobe.



All experiments were conducted in an ethical and humane way under approval from Liverpool University's Local Ethical Committee and under Home Office (UK) licensing. Common carp (*Cyprinus carpio*) were obtained and held as previously described [25]. During the experiment, individual fish were anaesthetised in aerated water dosed with benzocaine (1 ml/l of a 1 g/30 ml ethanol solution). Sterile physiological saline (0.9% NaCl; 0.1 ml) was injected 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 fish were returned to their home tank to recover from the anaesthesia. The control and treatment samples were collected at 1.5 h ($n=3$) and 3h ($n=6$) time points after the procedure since common carp show adverse behavioural signs *in vivo* at these time points [26]. The brain was dissected as a whole organ and fixed for 24 h in buffered 4% paraformaldehyde (pH 7.4). Transverse sections of approximately 1.5 mm were prepared from the brain and embedded in paraffin wax. Sections of 3–5 μ m thickness were cut onto Polysine™ glass slides (VWR International bvba, Leuven, Belgium) and stained with haematoxylin–eosin (HE) or dried for 1 week for use in ISH.

Primers used to construct the 190 base *vangl2* riboprobe for RNA-ISH were 5'-CGGTCCCTTTGGTGACTCTA-3' and 5'-CCTTGGATGTGGTAGCCGTT-3'. These primers were based on the carpBASE 2.0 consensus sequence of subgroup ID 679-1 (<http://legr.liv.ac.uk>), which shares 97% identity with the zebrafish *vangl2* mRNA sequence (GenBank accession no. AF428249). After ligation into a plasmid vector (TOPO TA Cloning® Kit pCR® II TOPO®; Invitrogen BV, Groningen, NL), in vitro transcription of a digoxigenin-labeled riboprobe was performed, using an SP6/T7 polymerase system (DIG RNA Labeling Kit; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Fig. 2. Common carp, telencephalon ('1' and '2' in Fig. 1) RNA-ISH with *vangl2* riboprobe, Papanicolaou's haematoxylin counterstain. (A) Expression of *vangl2* mRNA is mainly seen towards the dorsal, anterior section of the forebrain (AF: anterior forebrain; PF: posterior forebrain). (B) Closer view of the positive neurons in the area *dorsalis telencephali pars dorsalis* of the anterior forebrain. (C) Closer view of the positive neurons in the area *dorsalis telencephali, pars centralis* of the posterior forebrain.

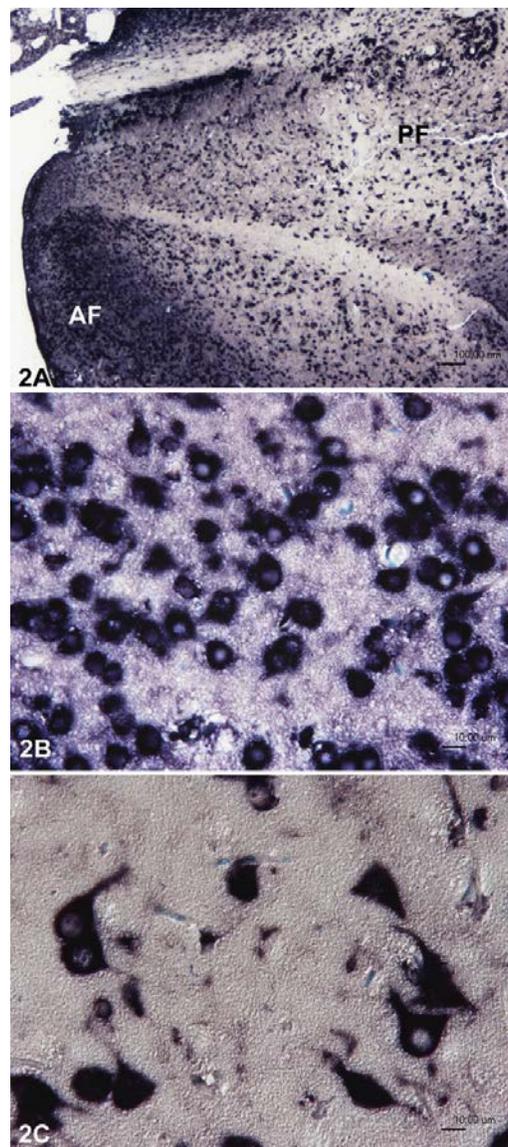
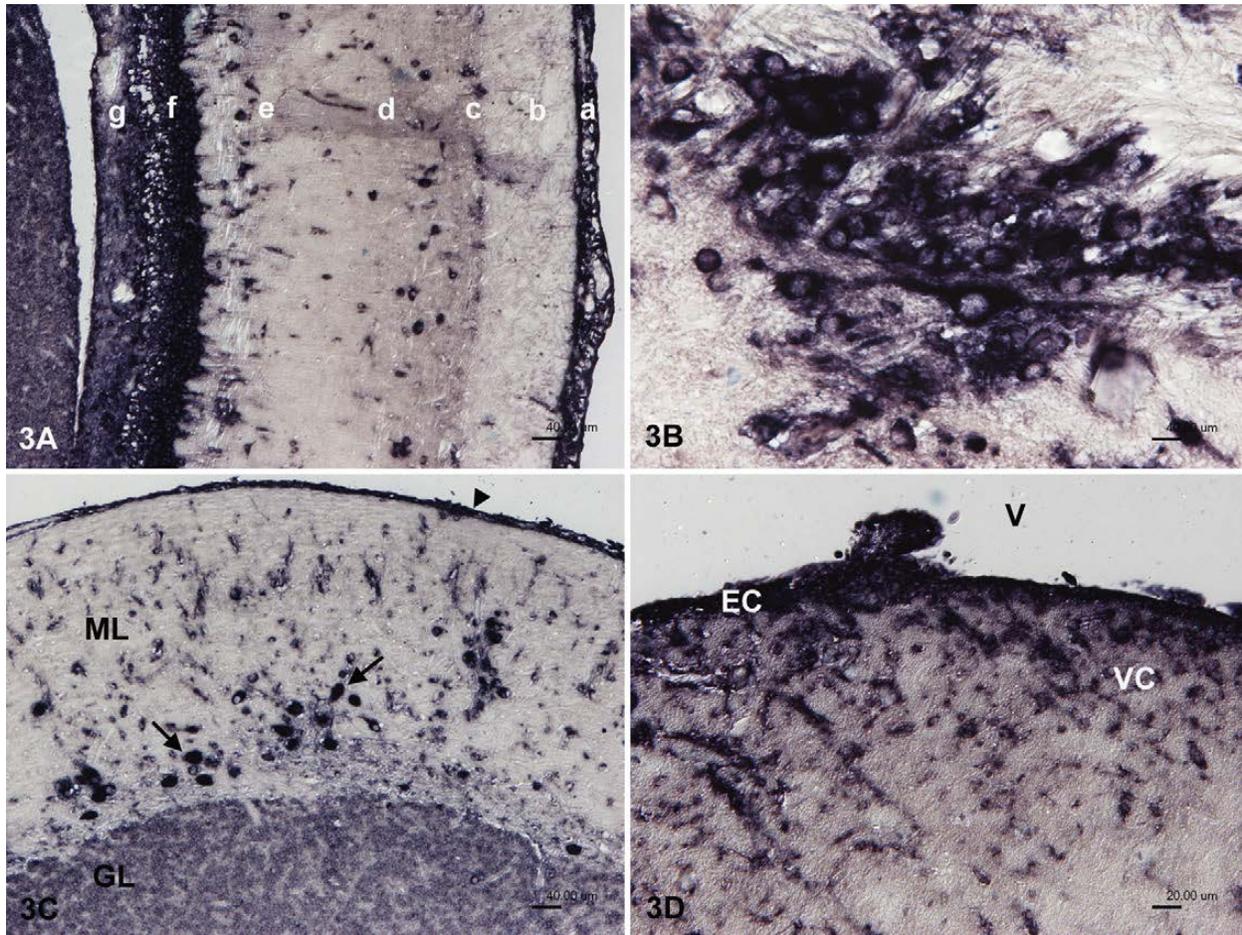


Fig. 3. Common carp, RNA-ISH with *vangl2* riboprobe, Papanicolaou's haematoxylin counterstain. (A) Midbrain, tectum mesencephali ('5' in Fig. 1). The organisation of the tectum is labeled (a) *leptmeninx/arachnoidea*, (b) *stratum fibrosum marginale* (molecular layer), (c) *stratum opticum*, (d) *stratum fibrosum et griseum superficiale*, (e) *stratum griseum centrale*, (f) *stratum album centrale*, (g) *stratum periventriculare*. (B) Midbrain, ventral thalamus ('4' in Fig. 1). All neurons exhibit strong *vangl2* mRNA expression. (C) Cerebellum ('9' in Fig. 1). Neurons in both the molecular layer (ML) and the granular layer (GL) as well as the large Purkinje cells (arrows) are positive for *vangl2* mRNA. Meningeal cells (arrow head) also exhibit a positive signal. (D) Ventricle (V) and *valvula cerebelli* (CV) ('8' in Fig. 1). Ependymal cells (EC) that boarder the ventricles express *vangl2* mRNA. Neurons in the *valvula cerebelli* are also positive.



ISH was conducted using the same method as described by Kipar et al. [16] using sense and antisense. Slides were deparaffinised and digested in proteinase K (1 µg/ml) at 37°C for 15 min. This was followed by postfix, acetylation and prehybridisation incubations. Hybridisation was performed at 37°C for 18 h and the riboprobe concentration was 1 µl/500 µl hybridisation mix. After hybridisation, slides were washed and stained with Anti-DIG-AKAP antibody (1:200) (Boehringer; Berkshire, UK) and nitroblue tetrazoliumchloride (Sigma-Aldrich; Poole, UK). A 10 s counterstain with Papanicolaou's haematoxylin (1:20 in distilled water) was performed. A positive reaction was represented by a dark purple to black cytoplasmic signal as seen in all figures. The number of *vangl2*-positive Purkinje cells was counted in the *lobus caudalis* of the cerebellum in an area measuring 160 µm × 160 µm (area '10' in Fig. 1). Control ($n = 5$) was compared to treatment ($n = 7$) using a two-tail unpaired Student's t -test using MINITAB software. The

number of non-Purkinje cells in the vicinity was also counted in three areas measuring 80µm×80µm for each animal and the mean was used as a representative value for the individual. These control and treatment mean values were also compared with a two-tailed unpaired Student's *t*-test.

Expression of *vangl2* in different brain regions: In the telencephalon, small neurons located towards the edges, and larger neurons centrally in the area *dorsalis telencephali, pars centralis* both exhibited positive signals for *vangl2* mRNA (Fig. 2). The different morphological characteristics of these cells were highlighted, as the positive signal was evident throughout the entire cytoplasm (Fig. 2B and C). The small neurons in the anterior region of the forebrain (Fig. 2B) were mainly round, with short, stubby cell processes. The larger, more sparsely arranged neurons of the central telencephalon (posterior forebrain) exhibited more abundant cytoplasm and longer processes (Fig. 2C).

Vangl2 expression was also observed in cells in all tectal layers of the carp midbrain (Fig. 3A). The few neurons present in the *stratum fibrosum marginale* and the *stratum opticum*, large neurons in the *stratum fibrosum et griseum superficiale*, neurons scattered through both the stratum griseum centrale and the *stratum album centrale* and all neurons of the innermost layer of the tectum, the *stratum periventriculare* exhibited a positive signal. The ventral thalamus which comprises a heterogenous population of small and larger neurons showed positive signals in both of these cell types (Fig. 3B).

In the cerebellum, Purkinje cells and neurons in the granular and molecular layer were positive for *vangl2* (Fig. 3C). Purkinje cells either form a monolayer between the granular and molecular layers or, in the *lobus caudalis*, are seen scattered within the molecular layer and their large cell bodies and dendritic trees are highlighted by the cytoplasmic *vangl2* signal (Fig. 3C). Meningeal cells, modified fibroblasts that cover the outer surface of the brain as part of the leptomeninx/arachnoidea, and ependymal cells, which are glial cells that line the ventricles and are believed to be involved in the directional movement of the cerebrospinal fluid, were both found to express *vangl2* (Fig. 3A, C, D).

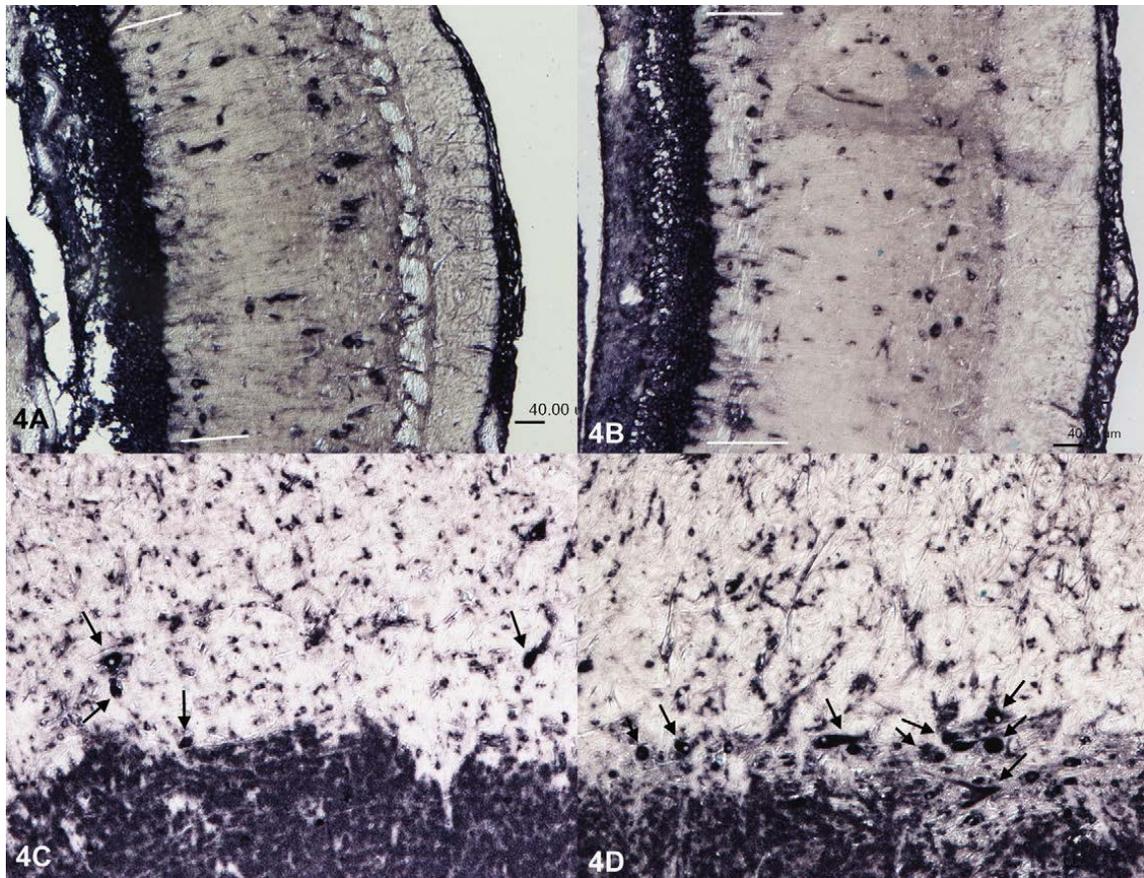
Effects of noxious stimulation: *Vangl2* mRNA was strongly expressed in neurons in the forebrain in both control and treatment fish. The pattern of *vangl2* expression in the tectum remained constant in control and treatment fish at both time points. There was an increase in the number of *vangl2*-positive neurons in the stratum *griseum centrale* in animals 1.5 h after treatment, but not 3 h after treatment (Fig. 4A and B). Expression of *vangl2* was observed in the hypothalamus in both control and treatment samples, without any distinct difference between both groups of fish. Expression of *vangl2* was detected in Purkinje cells of the cerebellum in control and treatment samples at 1.5 and 3 h, respectively.

The number of *vangl2*-positive Purkinje cells was counted in an area of the *lobus caudalis* of the cerebellum measuring 160µm×160µm for each condition. The number of *vangl2*-positive cells (either neurons or glia) that were not Purkinje cells was also counted in three areas measuring 80µm×80µm. There were significantly more *vangl2*-positive Purkinje cells, with 3.57 (±0.48) cells counted in treatment animals compared to 1.64 (±0.73) in control ($P = 0.031$; n control = 5, n treatment = 7) (Figs. 1, 4C, 4D). There were also noticeably more non-Purkinje cells present in this region in treatment animals, with 3.74 (±0.95) in control compared to 6.19 (±1.00); however, this difference was not statistically significant ($P = 0.109$; n control = 5, n treatment = 7).

Expression of *vangl2* mRNA was found throughout the brain of the adult common carp in treatment fish, which were subjected to a nociceptive input, as well as their respective controls at 1.5 and 3 h time points. Strong expression of *vangl2* was observed in the forebrain as well as in ependymal cells in all conditions that were examined. In the cerebellum of noxiously stimulated fish, the number of *vangl2*-positive Purkinje cells was increased when compared with controls. Investigations into the localisation of

vangl2 in the brain have hitherto concentrated on expression during embryogenesis [13,23,28,29]. The current study is the first to offer an analysis of *vangl2* mRNA expression in the mature carp brain.

Fig. 4. Common carp, (A and B) midbrain, tectum mesencephali ('5' in Fig. 1) RNA-ISH with *vangl2* riboprobe, Papanicolaou's haematoxylin counterstain. (A) Control animal; (B) treatment (1.5 h) animal. The *stratum griseum centrale* (layer between white lines, 'e' in Fig. 3A) exhibits an increased number of positive neurons in treated animals (1.5 h) compared to control animals. (C and D) Cerebellum ('10' in Fig. 1). (C) Control animal; (D) treatment (1.5 h) animal. The number of positive Purkinje cells (arrows) in treated animals (1.5 h) is higher than in control animals.



The *vangl2* gene was strongly expressed in the ependymal cells of the carp the function of *vangl2* in ependymal cells in the mature carp brain might be. One possibility is that expression of *vangl2* in the ependymal cells is needed to maintain the integrity of the blood–brain barrier. This suggestion is feasible because modulation of cytoskeleton elements, which can be achieved through *vangl2* activation of the JNK pathway [27], is important in maintaining the structure of the blood–brain barrier [17].

In the current study, in situ hybridisation showed that *vangl2* mRNA is prevalent in neurons in the telencephalon. This finding is in agreement with previous studies which demonstrated a function of *vangl2* in regulating gene expression in the forebrain, where it is essential for the expression of a number of anterior brain gene markers [23]. The homologous region of the telencephalon is the mammalian cerebral cortex, and in the developing mouse embryo, this region was found to express *vangl2* protein [30]. In mammals, the forebrain region is intimately associated with the perception and evaluation of noxious stimuli. For example, the forebrain regions of the prefrontal cortex, the anterior cingulate cortex and the

posterior parietal cortex are activated by the spatial discrimination of pain [22]. The intense expression of *vangl2* in the carp forebrain, along with the regulation demonstrated previously, means that it may be modulating the sensory perception through altered expression in the forebrain.

Expression of *vangl2* was detected in structures of the midbrain, which are known to be involved in mammalian pain processing. Constitutive expression of *vangl2* was detected in the thalamus, which is a major relay station of nociceptive information in mammals [19] and the hypothalamus, which coordinates the endocrine response to nociception [8]. Transcription of *vangl2* was also seen in all layers of the optic tectum, most densely in the *dura mater*, the *stratum fibrosum et griseum superficiale* and the *stratum periventriculare*. Most of the afferent fibres of the *stratum fibrosum et griseum superficiale* originate from retina [32], but this layer also receives input from the cerebellum and other tectal areas [10]. The cells of the *stratum periventriculare* are small monopolar neurons which give rise to dendrites in the *stratum fibrosum et griseum superficiale* [21]. Between 4% and 7% of the neurons in this layer are cholinergic [31], and a subset display Neuropeptide Y [33]. Therefore, the expression of *vangl2* in the tectum has the ability to affect the communication between the tectal layers and also interact with the cellular outputs from the tectum.

The expression pattern of *vangl2* in the carp brain is similar to some proteins that are involved in nociception. Proteins such as neuropeptide Y [12], the CB1 receptor [15] and the δ -opioid receptor [4], which modulate nociceptive signalling, are all highly expressed in the telencephalon of cyprinidae and salmonid fish [5,18,23], adding to the data which indicate that the telencephalon may be involved in nociceptive processing in fish [7]. Purkinje cells of the cerebellum expressed *vangl2*, and the number of cells stained by the *vangl2* probe increased in treatment fish compared to controls. The teleost cerebellum has previously been suggested to have a role in nociception as ablation of the goldfish cerebellum results in increased sensitivity to potentially painful stimuli [14]. Additionally, a high number of Purkinje cells were found to express the δ -opioid receptor mRNA [24]. Future *in situ* hybridization studies assessing whether these genes are co-localized with *vangl2* would be useful to determine whether particular cell types are responsible for controlling the response to nociceptive stimuli.

In the context of nociception and pain perception, *vangl2*-mediated cell signalling in the mature fish could be via the canonical Wnt pathway or the PCP pathway. The PCP pathway interacts with the cytoskeleton to control directionality of novel neurons [11], whereas the canonical Wnt pathway negatively regulates β -catenin to alter expression of target genes, some of which, for example nitric oxide synthase (NOS) [6], are involved in pain processing [1]. The Wnt/ β -catenin pathway is activated in the adult mammalian brain during neurogenesis [20] and it would be of interest to assess whether *vangl2* is able to modulate neurogenesis in the hippocampus through this pathway, as insight into this process may lead to novel treatments for chronic pain. A number of genes are activated during neurogenesis from 2 h onwards [9]. As *vangl2* was up-regulated at 1.5 and 3 h after the nociceptive event, then became down-regulated by 6 h [25], it is possible that *vangl2* is contributing to an early response which modulates the neurogenesis pathway.

The expression of *vangl2* mRNA was detected throughout the carp brain, particularly in the ependymal cells and the telencephalon. More Purkinje cells expressed *vangl2* after nociceptive treatment compared to controls. Due to its anatomical localization and regulation, *vangl2* should be further investigated as a novel target potentially involved in central nociceptive processing in higher vertebrates.

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