**Gtwnt-5** a member of the *wnt* family expressed in a subpopulation of the nervous system of the planarian *Girardia tigrina*

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**Abstract**

Wnt proteins are a family of highly conserved secreted glycoproteins that regulate cell-to-cell interactions during embryogenesis. They act as signaling molecules and take part in many crucial decisions throughout the development of organisms ranging from *Hydra* to human. We have isolated and characterized the expression of a member of the Wnt family, *Gtwnt-5* gene in the planarian *Girardia tigrina*. Planarians are free-living members (Class Turbellaria) of the Phylum Platyhelminthes. They are best known for their high regenerative capabilities. These organisms have an apparently simple central nervous system (CNS) from a morphological perspective, with cephalic ganglia in the dorsal anterior region and two ventral main nerve cords along the body. However, a large number of planarian neural genes have recently been identified and therefore it is possible to define different molecular and functional domains in the planarian brain. The present study shows expression of *Gtwnt-5* in a subpopulation of the whole CNS of intact organisms, being activated during regeneration. *Gtwnt-5* reveals a differential spatial pattern: the expression is preferentially found in the most external region of the CNS. In addition, a kind of iterative pattern has been observed at the ganglia level, suggesting that the planarian brain might not be a continuous structure but compartmented or regionalized. *Gtwnt-5* signal is also detected at the sensors of the worm: at the auricle level and all around the cephalic periphery. All these data provide us with a new neural marker for the planarian brain, and can be used to follow regeneration of the CNS.

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**Keywords:** Planarian; Regeneration; Central nervous system; wnt

1. Results and discussion

Genetics in *Drosophila* and *Caenorhabditis elegans*, biochemistry in cell culture, ectopic gene expression in *Xenopus* embryos, and knockouts in mouse have led to the conviction that Wnt proteins are essential for many of the decisions in development such as specification of cell polarity, cell fate identity and embryonic induction (Wodarz and Nusse, 1998). The genes included in the so-called XWnt-5A class are not able to duplicate the body axis after injection of mRNAs into ventral blastomeres of early *Xenopus* embryos, and instead they seem to play a role in controlling morphogenetic movements (Torres et al., 1996). With this work we report the isolation and characterization of a member of the Wnt family which clusters with the Wnt-5 class of wnt molecules.

Several genes have recently been isolated in the planarian central nervous system (CNS) (for review see Saló and Baguñà, 2002; Cebrá et al., 2002a,b; Pineda et al., 2002; Pineda and Saló, 2002). The planarian brain consists of two lateral lobes in an anterior-dorsal position, connected by a large anterior commissure and several lateral branches, eight or nine depending on the species, which connect the ganglia to the edge head sensors. More ventrally, the two main nerve cords extend from the ganglia to the tail of the worm (Morita and Best, 1966; Baguñà and Ballester, 1978; Rieger et al., 1991; Reuter et al., 1995; Agata et al., 1998). With this work we report the isolation and characterization of *Gtwnt-5*, a gene which is continuously expressed in both the cephalic ganglia and the ventral nerve cords of the organism, as well as in the sensory organs spread all around the periphery of the planarian, but mainly concentrated in the anterior part of the body. Compared with other neural genes like *Dugesia japonica Djotx-A, Djotx-B, Djotp* (Umésono et al., 1997, 1999), *D. japonica* and *Girardia tigrina Pax6A* genes (Pineda et al., 2002), *Gtsix3* (Pineda and Saló, 2002) and planarian brain EST’s collections...
(Cebrià et al., 2002a,b), it is possible to underline temporal and regional differences with Gtwnt-5 expression. During head regeneration Gtwnt-5 expression is activated at a mid stage and continuously expands through the whole regenerative process.

1.1. GtWnt-5 full sequence

We isolated a planarian cDNA fragment with homology to different wnt-5 genes by using degenerate polymerase chain reaction (PCR). The initial fragment was 140 bp in length and the complete cDNA sequence was obtained by 5’- and 3’-RACE (rapid amplification of cDNA ends) PCR. Fig. 1A shows the nucleotide and amino acidic sequences of the isolated Gtwnt-5 gene. The leader region includes the consensus translation sequence surrounding the ATG start codon: A/G CCATGG (Fig. 1A, in blue), which supports this first methionine is the starting translation point. Then
the Gtwnt-5 coding sequence starts with the ATG at position 7 and potentially codes for a protein of 335 amino acids that terminates with an amber codon at position 1012. The termination signal is followed by 268 bases of untranslated trailer sequence comprising the canonical poly(A) addition sequence, 13 nucleotides upstream of the insertion site. All wnt genes encode proteins with a nearly invariant pattern of 21–23 cysteines as well as some other conserved residues. BLASTX analysis defined Gtwnt-5 as a wnt-5 representative. With phylogenetic analysis using the neighbor-joining algorithm, a consensus tree is obtained which groups with a robust bootstrap value Gtwnt-5 with the Wnt-5 class (Fig. 1B). Previous analysis of wnt genes in Lophotrochozoans (Prud’homme et al., 2002) do not describe any wnt-5 gene, therefore Gtwnt-5 is the first identified in this clade.

1.2. Whole-mount in situ hybridization

1.2.1. Intact adults

The pattern observed in intact animals is as follows: the ganglia and the two main nerve cords expressing Gtwnt-5 in their external part, with no signal in the inner part (Fig. 2A). The distribution of the signal can be examined with detailed images of the ganglia, head periphery and other genes pattern comparison. Fig. 2B and C show Gtwnt-5 and GtPax6A pattern of expression at the ganglia level. Both are undoubtedly neural markers, but they reveal a differential
pattern, with \textit{GtPax6A} ubiquitous in the CNS. Transversal cryosections were performed with hybridized organisms, which corroborate the above mentioned type of signal. The internal region of the CNS remains negative for \textit{Gtwnt-5}, which obviously has neurons as revealed by the DAPI respective images (Fig. 2D,E and F,G). We speculate that the product of the gene could be distributed in a gradient manner, in an internal–external direction, with a maximum at the external part of the neural tube, the inner neurons remaining negative. In addition, \textit{Gtwnt-5} signal is present in the sensors located at the dorso/ventral border including the auricle (Fig. 2H, yellow arrowhead), the cephalic periphery (Fig. 2B, yellow arrowheads) and, in some cases, reaching the pharynx level (Fig. 2A, yellow arrowheads).

1.2.2. Regenerating planarians

Planarians are able to regenerate a complete head with brain, auricles and eye spots in 2 weeks at 17 °C (Reuter et al., 1995, 1996). We present data on the \textit{Gtwnt-5} expression pattern observed in regenerative organisms, ranging from 1 to 14 days of regeneration at 17 °C. Organisms of 1 and 2 days of regeneration showed no signal (results not shown). First signals appear at the third day of the process in an arch shape, which corresponds to the newly formed anterior commissure of the brain, in the blastema (Fig. 3A). Some other planarian neural genes like \textit{Djotx}, \textit{DjPax6A} and six EST clones are expressed from the beginning of the process, an indication that they may be implied in early determination of the ganglia and nerve cords (Umesono et al., 1999; Pineda et al., 2002; Cebrià et al., 2002a). According to the timing of their up-regulation in the new forming blastema, neural genes are classified as early, mid and late regeneration expression genes (Cebrià et al., 2002a). \textit{Gtwnt-5} might belong to the group of mid-regeneration genes: at 5 days of regeneration, an increase of \textit{Gtwnt-5} expression is also detected in the nerve cords located close to the wound, probably related to the remodeling of this region to the posterior part of the new cephalic ganglia (arrows in Fig. 3B). The sensory cells located at the edge of the head have just been differentiated and express \textit{Gtwnt-5} (yellow arrowheads). This expression is maintained throughout the whole regenerative process. At 7 and 10 days of regeneration, the signal at the postblastema region (arrows in Fig. 3C,D), is as strong as that observed at

Fig. 2. Whole-mount in situ hybridization of \textit{Gtwnt-5} in intact planarians. (A) Dorsal view of a whole intact planarian showing the expression in the central nervous system (arrows) and in the lateral anterior sensors from head to pharynx level (yellow arrowheads). (B) Higher magnification of the planarian head showing the expression in the intact cephalic ganglia (arrows) and the head sensors (yellow arrowheads). (C) Expression of \textit{GtPax6A} (arrows), a ubiquitous central nervous system molecular marker. (D–G) The transversal cryosections anterior to the eyes (D,E) or at the eyes level (F,G) showing \textit{Gtwnt-5} expression (D,F), together with Dapi staining (E,G) to localize the position of the nerve cell bodies, a circle of nuclei indicated by white arrows. In both sections \textit{Gtwnt-5} signal is located in the external region of the ganglia (arrows), dorsal in the top. (H,J) Higher magnification of the head dorsal view with the expression of \textit{Gtwnt-5} in the auricle (yellow arrowhead) and in the left cephalic lobe with a presumptive iterative pattern. a, auricle; e, eyes; ph, pharynx. Scale bars: 0.5 mm.
the blastema region (arrowheads in Fig. 3C,D), a fact that corroborates the epimorphic-morphallactic model of regeneration in planarians (Saló and Baguña, 1984, 2002): formation of new structures at the blastema region (in this case differentiation of the anterior ganglia) and reorganization of old structures at the post-blastema region (in this case, the nerve cords located close to the wound reorganize in order to be determined as the posterior part of the cephalic ganglia). In individuals of 12 and 14 days of regeneration, the activated Gtwnt-5 expression compared to previous stages decreases and reaches the basal levels, while the dorsoventral border maintains the Gtwnt-5 expression (yellow arrowheads). e, eyes; ph, pharynx. Scale bars: 0.5 mm.

Fig. 3. Whole-mount in situ hybridization of Gtwnt-5 during head regenerating planarians. (A) At 3 days of regeneration the first expression in the regenerative blastema can be observed with an arch shape that follows the new regenerated brain. (B) At 5 days of regeneration an increase in the Gtwnt-5 expression can be observed in the old nerve cords located close to the wound (arrows), also the new dorsoventral border where the sensor cells differentiate are expressing Gtwnt-5 (yellow arrowheads). (C) At 7 days of regeneration Gtwnt-5 expression labels the nearly regenerated cephalic ganglia; the yellow dashed line defines the border between blastema (new) and postblastema (old) regions, being the regenerated eyes (small brown dots) anterior to the border. (D) At 10 days of regeneration a similar pattern is maintained, with larger brain and eyes. (E,F) At later stages of regeneration, 12 and 14 days respectively, the activated Gtwnt-5 expression decreases until it resembles that observed in intact individuals. Fig. 2I is a detailed image of Gtwnt-5 expression at the ganglia level in organisms of late regenerative stages; a single focus plane is shown, but it is clear that not all the neurons express Gtwnt-5 as the pattern at this level seems to be distributed in a kind of iterative manner (see also Fig. 2H). In posterior or tail regeneration, Gtwnt-5 is activated later than 3 days in the new ventral nerve cords produced in the blastema and maintained throughout the whole process (Fig. 4A–C).

In conclusion, the results in intact animals suggest that G-twnt-5, a member of the Wnt-5 family, shows a continuous pattern of expression in the anterior sensors located at the dorsoventral border and in the CNS. It is a different pattern compared to previously described cephalic genes, and can be considered a new (intermediate regenerative stage) neural marker of the planarian CNS, with an external-internal gradient of distribution.

2. Experimental procedures

2.1. Species

The planarians used in this study belong to an asexual race (class A; Ribas et al., 1989) of Girardia tigrina. They were collected near Barcelona, maintained in spring water and fed with chicken liver twice per week. Planarians, 9–10 mm long, were cut transversally according to Saló and Baguña (1984) and left regenerating in Petri dishes with spring water in the dark at 17 °C.
2.2. **Gtwnt-5 cDNA cloning**

RNA extractions were performed according to the protocol of the commercial kit Trizol (Gibco BRL). The SMART RACE cDNA Amplification kit (Clontech) and the GeneRacer kit (Invitrogen) were used to prepare the specific cDNA needed to perform the PCR–RACE reactions. We meant to complete the 3' and 5' ends of an initial fragment of 140 bp obtained by degenerated PCR. The two degenerated primers were: DWnt-1 forward: 5'-TGG AA(AG) TGG GG(AGCT) GG(AGCT) TG(CT) (GT)A-3' and DWnt-3 reverse: 5'-ATA (AT)CC (ACGT)C(GT) (AT)CC (AG)CA (AG)CA (AGCT)A-3'. The PCR conditions were: denaturation step, 94°C 30 s; hybridization step, 67°C 30 s; and elongation step, 72°C 2 min. Number of cycles: 36. The specific primer designed for 3'-RACE PCR was: w3': 5'-GGGTGTGGAGATAATTTAAGATATGCC-3'. A total of 35 cycles of PCR was performed under these conditions: denaturation step, 94°C 30 s; hybridization step, 67°C 30 s; and elongation step, 72°C 2 min. The specific primer designed for 5'-RACE PCR was: w5': 5'-GCA-GATTGACTAAAATGATTCCAG-3'. A total of 35 cycles of PCR was performed under these conditions: denaturation step, 94°C 30 s; hybridization step, 63°C 30 s; and elongation step, 72°C 2 min. We obtained two clones of 200 and 907 bp, respectively, from the 3'-RACE and one clone of 399 new base pairs of codifying region, reaching the first methionine, from the 5'-RACE. All clones were subcloned in pBluescript + SK (Stratagene), digested with Sma I restriction enzyme and dephosphorylated. With the sequencing (ABI PRISM, Perkin Elmer) and after BLASTX analysis, all clones turned out to be homologous to the wnt-5 genes found in other species. We named it Gtwnt-5, with 1005 bp of codifying region and 268 bp of the trailer non-codifying region of the gene. The codifying region was used as a basis for the phylogenetic analysis. The previous alignment was performed after removing some parts of the molecule: (a) the first 23 amino acids, (b) the region including the last nine conserved cysteines, and (c) ambiguous regions from which homology cannot be assured, like some large insertions which were only present in a few of the species. Distance matrix was inferred with the PROTDIST program from PHYLIP set, using the Dayhoff PAM matrix; phylogenetic tree was inferred with the FITCH program, from PHYLIP set, using the Fitch–Margoliash algorithm.

2.3. **In situ hybridization**

The codifying region from position 400 to position 1011 was used to synthesize a riboprobe labeled with UTP-digoxigenin, using the in vitro Roche labeling kit. The whole-mount in situ hybridizations were performed with intact and regenerative animals at different regenerative stages according to Agata et al. (1998), with some modifications: proteinase K (20 µg/µl), between 8 and 10 min depending on the size and stage of regeneration; hybridizations of 36 h at 55°C with a final riboprobe concentration of 0.04 ng/µl. Images were obtained using Leica DMLB 10x, 20x and 40x objectives, and a digital camera (Nikon COOLPIX 995).

The histological cuts were done at −24°C with Bright...
Clinicut. The preparation of the slides as well as the sections was done according to Cebrià (2000).

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