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Early Development of the Trunk Neural Crest Cells in the Egyptian Cobra *Naja h. haje*

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Abstract

Background: Trunk neural crest cells (TNCC) are representing a model for epithelial to mesenchymal transition, this correlates the importance of studying the migration of these cells to cancer metastasis. Reptiles are unique group of animals being very morphologically diverse and their close position to synapsid leading to mammals. Recently, more publications focused on the migratory behavior of trunk NCC during embryonic development of squamates. Only one colubrid snake has been studied so far regarding the NCC migration.

Results: Here we follow the migratory behavior of TNCC with HNK1 in the elapid snake *Naja h. haje* from early stage to 14 days postoviposition. Comparing the colubrid snake with the Egyptian cobra showed that both snakes overall follow the same TNCC migratory pathways of both birds and mammals by following the rostral and avoiding the caudal portions of the somites.

Conclusions: First, TNCC intra-somitic migration as observed in turtles supports a contributing role for TNCC to scale precursors. Second, our observation of significant numbers of migrating TNCC in the intersomitic pathway suggest interesting evolutionary differences. Together, our present results of the Egyptian cobra in combination with those on a colubrid and turtle supports intersomitic TNCC as a unique reptile phenomena.

Keywords: Neural Crest cells; Delamination; Mesonephri; Enteric nervous system

Introduction

Studying and comparing developmental events between different species helps to explore the developmental dynamics of organs and structures and to evaluate the evolutionary transitions across groups. Reptiles are a unique group of animals, being very morphologically diverse and because the close position of synapsids leading to mammals (Vitt and Caldwell, 2014), this makes the group very interesting to follow the development of their nervous system. Among reptiles, squamates are the good model for comparative developmental dynamics study, being very diverse in cranial anatomy and kinetics (Khannoon and Evans, 2015).

Neural crest cells (NCC) are an early migratory population that give rise to very different cells: sensory neurons, glia, melanocytes and chromatophores of the integument, osteocranial components, and specialized cells in glands (Baggiolini et al., 2015; Le Douarin and Dupin, 2018; Trentin et al., 2004). There are few studies on the NCC focused on squamates: chameleon, turtles, crocodile and snake (Diaz et al., 2019; Goldberg et al., 2020; Hou and Takeuchi, 1994; Kundrat, 2008, 2009; Reyes et al., 2010). These studies show that trunk NCC (TNCC) migratory patterns in non-avian reptiles are highly conserved across sauropsids, and the pattern is largely conserved between reptiles. However, while TNCC in birds make a robust stream of migrating cells along the ventromedial pathway (Giovannone et al., 2015), in the colubrid *Lampropeltis getula californiae* was unique in having a much smaller number of TNCC along this pathway (Reyes et al., 2010). On the other hand, turtle embryos showed some unique features that are not found in other amniotes: TNCC forming a line along the trunk lateral mesoderm, and at late stages the migrating TNCC used the medial pathway of the somite (Goldberg et al., 2020).

Snakes are good model to study delamination and differentiation of NCC, because they have a very long body with many developing somites. Additionally, grow of the tail and the different coils enable scientists to have a simultaneous possible observation of both delamination and differentiation of NCC. It is of importance to investigate a different snake from different families of serpents to test whether the same pattern of TNCC migration is conserved within one clade. Here we follow TNCC migration in the elapid snake *Naja haje haje*. This snake has been recently considered in studying the embryonic table (Khannoon and Evans, 2014, 2015). It is more feasible to follow NCC migration in an established-embryonic table model. Comparison between this elapid snake and the previously studied colubrid snake *L. getula californiae* (Reyes et al., 2010) should give us more understanding on the pattern of delamination and migration of TNCC within serpents.

Methods and Materials

Naja haje haje embryos

Adult gravid female *Naja haje haje* were collected from Fayoum in June and transferred to cages in a similar condition to the field. Fertilized eggs (n = 50) were collected and were placed in plastic boxes (at 85–90% moisture). They were incubated at a temperature of 30 °C. At this temperature, hatching occurs in approximately 51–54 days. At early stages, eggs were opened every day, and at older stages at every other day. Embryos were investigated in phosphate buffered saline (PBS), and they have been extracted from the surrounding extra embryonic membranes. Embryos were examined under a stereomicroscope before fixation in 10% buffered formalin and 4% paraformaldehyde (4% PFA). The percentage of infertile embryos and hatching success can be checked in (Khannoon and Evans, 2014, 2015). All the animal work was approved and permitted by Fayoum University, Faculty of Science.

Pre-treatment of Embryos

The embryos were washed several times in PBS after fixation. Afterwards they were dehydrated in a series of washes in Ethanol of the following concentrations: 25%, 50%, 70%, 90%, and 100% for 60 minutes each until they sunk down in the vial. Two more washes in ethanol were done afterwards for 30 minutes each. Two washes, 10 minutes each, were done with Histosol/xylene using gloves under a hood following the washes. They were then paraffinized in an oven.

Agar Preparation for vibratome of embryos

Embryos were fixed into an agarose solution made from 4% agarose and PBS. 4% agarose was put into 100mL of PBS in an Erlenmeyer flask and was mixed until it was homogeneous. It was then microwaved until all the bubbles were gone. Be cautious of the agar erupting from the Erlenmeyer flask – as a precaution, microwave in 15 second increments, stir and then continue microwaving. If there are any remaining bubbles in the center of the flask, the flask can be put on a hot plate. The agar was then let to cool for a small amount of time to avoid cooking the embryo. The embryo was then placed in a weighing boat in the orientation that it was going to be cut in and the agar was poured into the weighing boat and the embryo was further positioned in the orientation it was going to be cut. Make sure to lift the embryo off the floor of the weighing boat to center it. Once the agar solidified, a cube around the embryo was cut to begin vibratome sectioning.

Vibratome Sectioning

Before cutting the agarose block with the embryo submerged in it, the Vibratome was first assembled. A piece of tape was placed on the specimen disc where a small amount of Crazy

Glue was poured onto the tape. This ensures easy removal of the embryo to continue sectioning the next day if needed. The agarose cube with the embryo is then placed on the glue in the orientation it is going to be sectioned in. The specimen disc with the embryo glued on is then screwed onto the buffer tray which is then placed in the ice bath portion of the vibratome. The blade is placed in the blade holder and then screwed onto the vibratome. At this point, cold PBS buffer is poured into the buffer tray and ice is poured around the buffer tray to ensure that the PBS is maintained at a cold temperature. The vibratome machine is turned on and the sections are cut at 65 μ m.

Primary and Secondary Antibody Stain

The floating sections were blocked in a humidified chamber overnight in PBS with 1% Triton and 10% goat serum. Next day primary antibodies were added at 1:250 (HNK1 or Sox9) in PBS. The following day, the sections were washed three times each for 10 minutes each in PBS. The secondary antibody stain was added (Alexas anti-mouse IgM-594 or 633, and anti-rabbit 488). Next day sections were washed extensively in PBS and imaged in AxioImager Zeiss microscope.

Results

As done in the past, we used the HNK1 antibody to label the migrating neural crest cells (Cebra-Thomas et al., 2007; Giovannone et al., 2015; Goldberg et al., 2020; Reyes et al., 2010). We began with an HNK1 wholemount in a 2.5 coil cobra embryo (Stage 1/2: 4-5dpo) (Fig.1). In this early embryo we could see the beginning of condensing dorsal root ganglion (DRG) in the 1st coil (arrows in Fig.1A) compared with coil end, where we did not observe segmental migration of TNCC (arrowhead in Fig.1A). The next segment in the 1st coil, showed migrating TNCC in their classic segmented manner along the rostral portion of somites (arrows in Fig.1B). Towards the beginning of the last coil, we observed very few migrating TNCC, though there were many on top of the NT (arrow in Fig.1C) and many too scattered on the ectoderm (arrowheads in Fig.1C). A higher magnification at mid-trunk level (2nd coil), showed that there are quite a number of migrating TNCC segmentally on the rostral side of the somites and beginning to condense into sympathetic ganglia (arrows in Fig.1D), but we also saw a large number of migrating TNCC that were not migrating in segmental streams on rostral somites but beneath the ectoderm (arrowheads in Fig.1D). A higher magnification at more rostral levels (1st coil) we could clearly see condensed DRG (arrows in Fig.1E) and few scattered cells that were beneath the ectoderm (arrowheads in Fig.1E, F).

The key findings from this embryonic stage show that: 1) the DRG develop quickly in the cobra as we observed in a colubrid and recently in a turtle (Goldberg et al., 2020; Reyes et al., 2010). 2) There was a large number of migrating TNCC along the trunk that did not seem to follow the ventromedial segmented pattern and above the NT. These cells could be melanocyte precursors, though in chicken and turtle these cells delaminate much later in development (Giovannone et al., 2015; Goldberg et al., 2020). Which poses the question about their possible contribution to the snake scales as we know that TNCC contribute to turtle shell (Goldberg et al., 2020).

An older *Naja haje* embryo (Stage 2/3; 7dpo) wholemounted with HNK1 showed fewer migrating TNCC streams in the 1st – 2nd coils, most HNK1-positive cells were condensing DRG in these two coils (arrows and arrowheads respectively in Fig.2A-D). A dorsal view on 1st coil showed condensed DRG (arrows in Fig.2E), while in the 3rd (last) coil the migrating TNCC were seen along the rostral side of the somites (arrowheads in Fig.2A, F). At the 2nd coil, streams of TNCC are migrating between somites (red arrow in Fig.2A).

Sections of this embryo show this progressive migratory development of the TNCC along the rostrocaudal axis of the cobra. In the 1st and 2nd coils the condensed DRG were fully formed (white arrows in Fig.3A-D, J, K). But at the 3rd coil (white arrows Fig.3E-F) and coil end (white arrows in Fig.3G-H) we could observe streams of migrating TNCC before beginning to condense in DRG or sympathetic ganglia (SG). What was striking in these caudal sections was the presence of TNCC entering ventral mesoderm towards what would be the gut (white arrowheads

in Fig.3E-G). We also observed TNCC entering lateral mesoderm (red arrows in Fig.3E, G, H) and that the ectoderm was also stained with HNK1 (red arrows in Fig.3 A-H).

The key findings from this embryonic stage are: 1) In short time, the DRG seemed finished condensing into ganglia. 2) The TNCC development of the cobra progresses in a smooth rostral to caudal manner as we observed in a colubrid king snake (Reyes et al., 2010). 3) Our observations open the possibility that cobra TNCC may contribute to the gut enteric nervous system in contrast to other amniotes (Burns and Le Douarin, 2001; Le Douarin, 2008; Zuhdi et al., 2015). 4) The presence of TNCC entering the lateral mesoderm as in turtles may contribute, that may contribute to snake scale formation (Cebra-Thomas et al., 2007; Gilbert et al., 2007; Goldberg et al., 2020; Rice et al., 2016). These migrating TNCC were not part of the ventrally migrating cells that will form the peripheral nerves, since they were a separate group of cells.

Finally, we looked at an older stage (early stage 4/5; 16dpo), when no more coils are added, and the head is fully distinguished. We could not do wholemount immunostaining of this old embryo, but we looked at HNK1 labeling in combination with Sox9, another marker of NCC as well as chondrocyte stem cells (Li et al., 2002; Mori-Akiyama et al., 2003; Spokony et al., 2002). Longitudinal sections showed that HNK1 strongly labeled the DRG, sympathetic ganglia and nerves at this stage (Fig.4A-C). On the other hand, Sox9 labeled the chondrocytes forming the cartilage in this embryo (green arrows in Fig.4A-C). The spinal cord was also labeled with HNK1 in the peripheral regions while the ventricular zone was positive for Sox9 (green arrows in Fig.4D-E), known to label oligodendrocyte precursors (Finzsch et al., 2008). Interestingly we found extensive HNK1 labeling between the mesonephros (arrows in Fig.4F), suggesting that these cells could be the ones that will give rise to adrenergic cells (Howard and Bronner-Fraser, 1985). Longitudinal sections of this embryo showed the precision of DRG segmentation in the cobra (Fig.3J-K). A closer look at the more developed skin in this embryo, showed HNK1-positive cells in the ectoderm and beneath the skin (red arrows in Fig.4A-H). We do not know which type of cells these next to skin cells are, but they could be part of the sensory cells in the snake like Merkel, Ruffini or Pacinian (Jackson, 1977).

The key findings from this embryonic stage 5 are: 1) We observed HNK1 positive cells under the ectoderm or in the epidermis, that could be melanocytes or sensory skin receptors, respectively (Giovannone et al., 2015). 2) The continuous presence of HNK1-positive cells in the mesonephros, which could be the adrenergic precursors of NCC origin (Ayer-Le Lievre and Le Douarin, 1982).

Finally, we looked in more detail at three phenomena we observed. First the intersomitic pathway in older cobra segments, since this is a more unusual pathway for amniote TNCC. A closer look at the wholemount of stage 2/3; 7dpo confirmed the presence of a significant number of TNCC migrating between somites (arrowheads in Fig.5A). Second, we observed intermingling of migrating TNCC next to the dorsal aorta. Sections of stage 4/5 embryo confirmed that along the dorsal aorta, migrating TNCC were intermingling between rostral and

caudal streams as described for chicken by Kulesa (Kasemeier-Kulesa et al., 2005). Third was the presence of significant number of migrating TNCC between the ventro-medial/DRG pathway, which we proposed may contribute to scale formation (green arrows in Fig.5D) (Goldberg et al., 2020).

Discussion

We provide the migration of the TNCC in the elapid snake *Naja haje haje* using the NCC marker HNK1. We observed a similar pattern to other amniotes, underscoring how conserved trunk NCC migration is across amniotes. Still we found some unique features in the Egyptian cobra. One of these features characteristic to the Egyptian cobra is the presence of large number of migrating TNCC above the NT. Similar cells were recorded in the colubrid snake *Lampropeltis getula californiae* and the turtle *Trachemys scripta* (Goldberg et al., 2020; Reyes et al., 2010), but they were later in development and were in segmented pattern. Here in *N. haje haje*, they are not following a segmented pattern and are scattered above the NT. These cells could be melanocyte precursors. Additionally, due to their early appearance relative to those in chicken and turtle we think that these cells might be sharing in scale development of the Egyptian cobra. The same role has been suggested in the turtle shell (Goldberg et al., 2020).

Our observation of intersomitic TNCC in the Egyptian cobra has only been observed before in the colubrid snake and the turtle (Goldberg et al., 2020; Reyes et al., 2010). In comparison to birds and mammals, it has been proposed that the intersomitic migration is a derived character for reptiles (Reyes et al., 2010). Our findings for Egyptian cobra reinforce this hypothesis.

The presence of distinct HNK1-positive cells in mesonephros, later in development than those we observed in the colubrid snake (Reyes et al., 2010) or in the turtle (Goldberg et al., 2020) could represent immature chromaffin cells (still positive for HNK1) or differentiated cells. Nevertheless, our finding continues to support a role played by NCC in developing the chromaffin adrenal cells of earlier vertebrates, and neither excludes the possibility that TNCC join the development of mesonephroi itself at least partly as suggested in the past (Gabe, 1970; Rupik, 2002). Despite the difference in timing, both colubrid snake and the Egyptian cobra showed the role of TNCC in development of chromaffin cells of the adrenal gland. Detailed study on the role of NCC in developing kidney is needed to give detailed discussion on the exact roles of NCC in this area in reptiles, and amniotes generally.

We would like to propose that the early and/or late migrating TNCC in large numbers at stage 1-3 of the Egyptian Cobra could be contributing to cobra's scale precursors, while the HNK1 cells observed at later stages 4/5, are likely melanophores or sensory skin receptors. It has been proposed recently that the TNCC migrating between DRGs and through mesodermal regions are TNCC that have the capacity for osteogenesis and therefore are able to form plastron in turtles and hypothetically, scales in snakes (Goldberg et al., 2020; Moustakas-Verho et al., 2017; Moustakas-Verho et al., 2014). We propose that TNCC may contribute to scales because the large number of cells migrating under the ectoderm could not be only skin sensory precursors, their density is higher than the observed density of these cutaneous structures in mature snakes (Jackson, 1977; Vieira et al., 2016). The observed contribution of TNCC to turtles plastron and carapace seemed to concentrate on the most external layer (non-osteogenic) that is similar to snake scales: changes/shedding with animal's growth or after injury, with

chromatophores to give it coloration and is not composed as one large segment, and has a true corneal layer composed of separate cells from the underlying bone (Cao et al., 2019).

The spindle shaped DRGs observed here in the Egyptian cobra are like those identified in the colubrid, chameleon and gecko (Diaz et al., 2019; Reyes et al., 2010). On the other hand, turtle DRGs are still larger in proportion to the somite segment, covering more area of somite segments and oval in shape. These observations in the elapid cobra plus what we also observed in the colubrid in comparison with turtles, might be related to the hypothesis on the role of DRGs and the evolution of homeothermy (Goldberg et al., 2020; Reyes et al., 2010). Another explanation posed that this difference in DRGs places turtles a distance from other sauropods, closer to Archosauria (Goldberg et al., 2020). After our observations on the cobra TNCC, we think that DRG size differences is more likely due to phylogenetic rather than an active lifestyle-related phenomenon.

One finding from the present study is the quick development of DRG from migrating TNCC. This phenomenon was also identified in the colubrid snake (Reyes et al., 2010) and the turtle (Goldberg et al., 2020) in contrast to chicken (Giovannone et al., 2015). Another character is the rapid rostrocaudal progression of TNCC migration and development observed for the Egyptian cobra was the same of the colubrid snake.

Conclusions

Comparing the colubrid snake with the Egyptian cobra showed that both snakes overall follow the same TNCC migratory pathways of both birds and mammals by following the rostral and avoiding the caudal portions of the somites. However, two main findings come from this study. First, TNCC intra-somatic migration as observed in turtles supports a contributing role for TNCC to scale precursors. Second, our observation of larger numbers of migrating TNCC in the intersomatic pathway suggest interesting evolutionary differences. This intersomatic migration has been observed in earlier stages of chicken embryos (Serbedzija et al., 1989, 1992) and in the chameleon (Fig.2 in (Diaz et al., 2019)). In the past we had suggested that the large number of intersomatic TNCC is a derived character of snakes (Reyes et al., 2010), or could be a lost developmental aspect in birds and mammals. Our present results of the Egyptian cobra in combination with those on a colubrid supports it as a unique snake character.

Figure legends

Figure 1. Wholemount of *Naja haje* embryo stage 1/2; 4-5dpo stained with HNK1 and DAPI.

A *Naja haje* embryo (st.1/2) was stained with HNK1 (red) and DAPI (blue). **A)** First and last coil (1st and 3rd) of the cobra embryo. Arrows point to condensing DRGs and arrowhead to very few HNK1 cells at the tail end. **B)** Second coil showed segmentally migrating trunk NCC strongly stained with HNK1 (arrows). The inset shows the whole embryo morphology before wholemount immunostain. **C)** The last, 3rd coil showed only scattered HNK1 cells beneath the ectoderm (arrowhead) as well as large number of trunk NCC delaminating from dorsal neural tube (arrow). **D)** A higher magnification of 2nd coil showed segmental migration and the beginning of sympathetic chain (arrows), while arrowheads point to scattered trunk NCC beneath the ectoderm. **E)** A higher magnification of the 1st coil shows condensed DRG (arrows) and few scattered trunk NCC beneath the ectoderm (arrowhead). **F)** A higher magnification of the most rostral 1st coil shows unique HNK1-positive cells beneath the ectoderm (arrowhead).

Figure 2. Wholemount of *Naja haje* embryo stage 2/3; 7dpo stained with HNK1 and DAPI.

A *Naja haje* embryo (st.3/4) was stained with HNK1 (red) and DAPI (blue). **A)** Shows the 3 coils of the embryo Coil 1 is the coil closest to the head of the embryo while coil 3 is tail end. **B-D)** Show a more uniform HNK1 staining of migrating TNCC throughout the 3 coils (arrowheads). **D)** The 1st coil shows less prominent HNK1 stain of TNCC compared to coils 2nd-3rd (**B-C**), but we can still observe robust HNK1 the vagal portion of the embryo (arrow). **E)** A dorsal view of cobra embryo at this stage shows condensed DRG (arrows). **F)** Shows TNCC from a rostral (red arrow) to caudal point of view and a reduction in signal can be seen in the caudal-most portion of the embryo.

Figure 3. *Naja haje* embryo stage 2/3; 7dpo sections cut by vibratome and stained with HNK1 and DAPI.

Vibratome sections are shown in a rostral to caudal progression (Panels **A-H**). The DRG's can be seen taking shape the closer at more rostral sections in 1st and 2nd coils (Panels **A-C**). At more caudal sections DRGs are not clearly condensed (arrows in **E-H**). **I)** Shows the wholemount image of the embryo before it was vibratomed and the line shows the level of the sections shown here. **J-K)** Show two sections from 1st coil with condensed DRGs lateral to the neural tube

(arrows). Some of the more caudal sections seem to have TNCC moving into the gut (white arrowheads in **E-H**). Red arrows in **E, G-H** show HNK1 TNCC in lateral mesoderm.

Figure 4. *Naja haje* embryo stage 4/5 stained with HNK1 and Sox9.

Sections through an older embryo (with maxilla and mandible already defined and 3.5 coils) showed a well-developed peripheral nervous system. **A-B**) Longitudinal sections in second coil showed HNK1 (red) labeling DRGs (green, white arrow), nerves and notochord (white arrowhead), while Sox9 labeled cartilage (green arrow). **C**) Transverse section of 2nd coil showed HNK1 stained sympathetic ganglia (SG white arrow) and Sox9 stained cartilage (green arrows). **D-E**) Sections by 3rd coil showed spinal cord stained with HNK1, DRG and dorsal root (white arrow in **D**). But Sox9 besides staining cartilage, labeled a group of cells in the midline of spinal cord (green arrowhead), likely oligodendrocyte precursors. **F**) A longitudinal section by the mesonephros showed that these were labeled with Sox9 while HNK1 cells are visible between the tubules (arrows). **G-H**) Brightfield images of the cobra embryo before sectioning. **I**) HNK1 labels the external layer of the ectoderm (white arrowheads), while cells and nerves are visible with HNK1 beneath it (white arrows). Spinal nerves and vertebrae are stained with HNK1 and Sox9 respectively.

Figure 5. Intersomitic TNCC migration within the Egyptian Cobra *Naja haje*.

A) Higher magnifications of Fig.2 embryo (stage 3/4) show the presence of intersomitic HNK1 migrating cells (red arrowheads). **B-D**) Longitudinal sections of this same embryo along 2nd coil show extensive ventral, next to dorsal aorta intersomitic migration (white arrows). **D**) Shows extensive staining of ectoderm with HNK1 (white arrowheads) as well as the unique intersomitic HNK1 cells between the rostrally migrating TNCC (green arrows).

Declarations:**Ethics approval and consent to participate**

All the animal work was approved and permitted by Fayoum University, Faculty of Science.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions:

EK collected *Naja haje haje* embryos and wrote manuscript. CA did wholemounts and sectioning of embryos, wrote portions of manuscript. MEdB did wholemounts and wrote manuscript.

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Consent for publication

Not applicable

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Figures

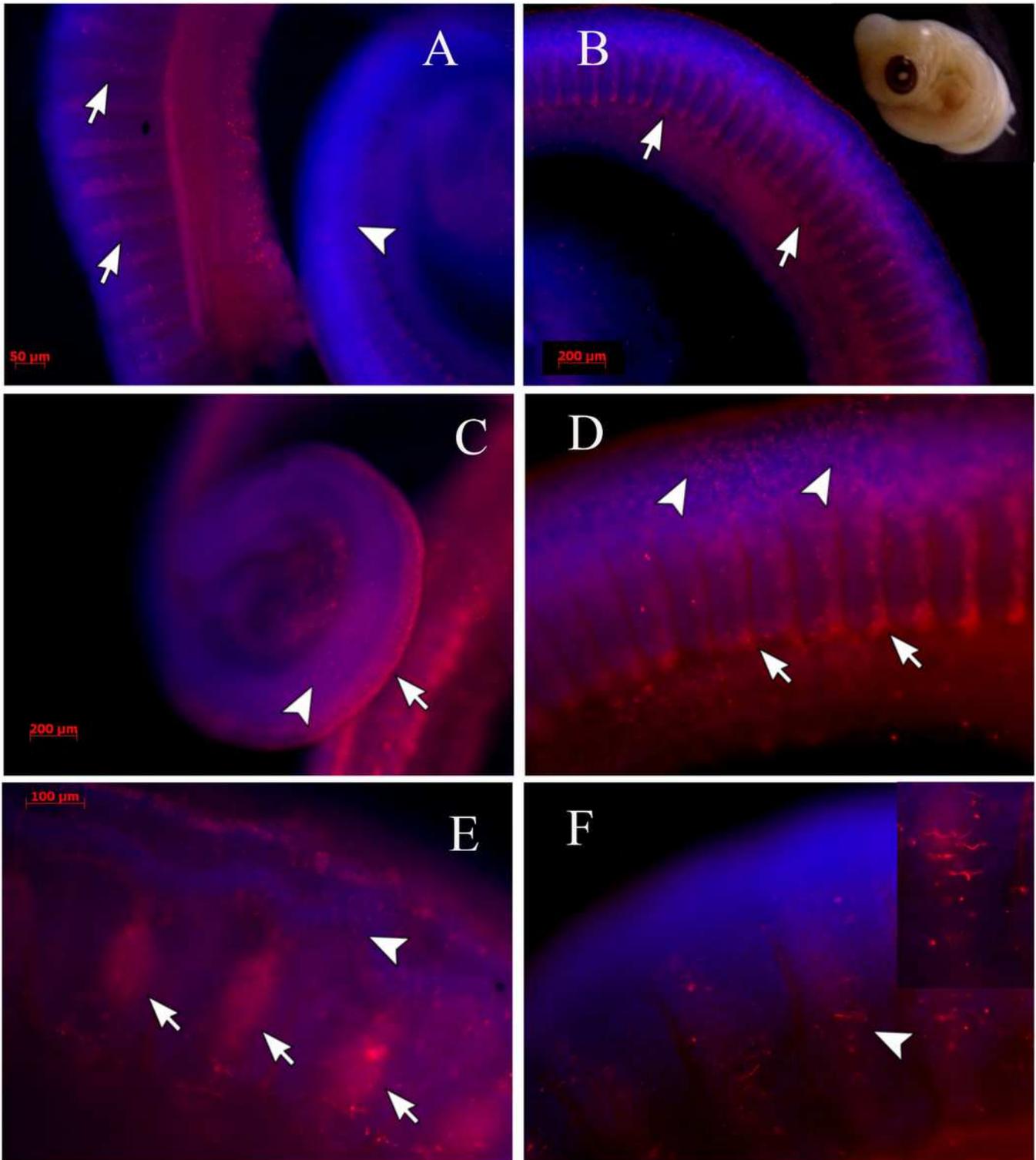


Figure 1

Wholemount of *Naja haje* embryo stage 1/2; 4-5dpo stained with HNK1 and DAPI. A *Naja haje* embryo (st.1/2) was stained with HNK1 (red) and DAPI (blue). A) First and last coil (1st and 3rd) of the cobra embryo. Arrows point to condensing DRGs and arrowhead to very few HNK1 cells at the tail end. B)

Second coil showed segmentally migrating trunk NCC strongly stained with HNK1 (arrows). The inset shows the whole embryo morphology before wholemount immunostain. C) The last, 3rd coil showed only scattered HNK1 cells beneath the ectoderm (arrowhead) as well as large number of trunk NCC delaminating from dorsal neural tube (arrow). D) A higher magnification of 2nd coil showed segmental migration and the beginning of sympathetic chain (arrows), while arrowheads point to scattered trunk NCC beneath the ectoderm. E) A higher magnification of the 1st coil shows condensed DRG (arrows) and few scattered trunk NCC beneath the ectoderm (arrowhead). F) A higher magnification of the most rostral 1st coil shows unique HNK1-positive cells beneath the ectoderm (arrowhead).

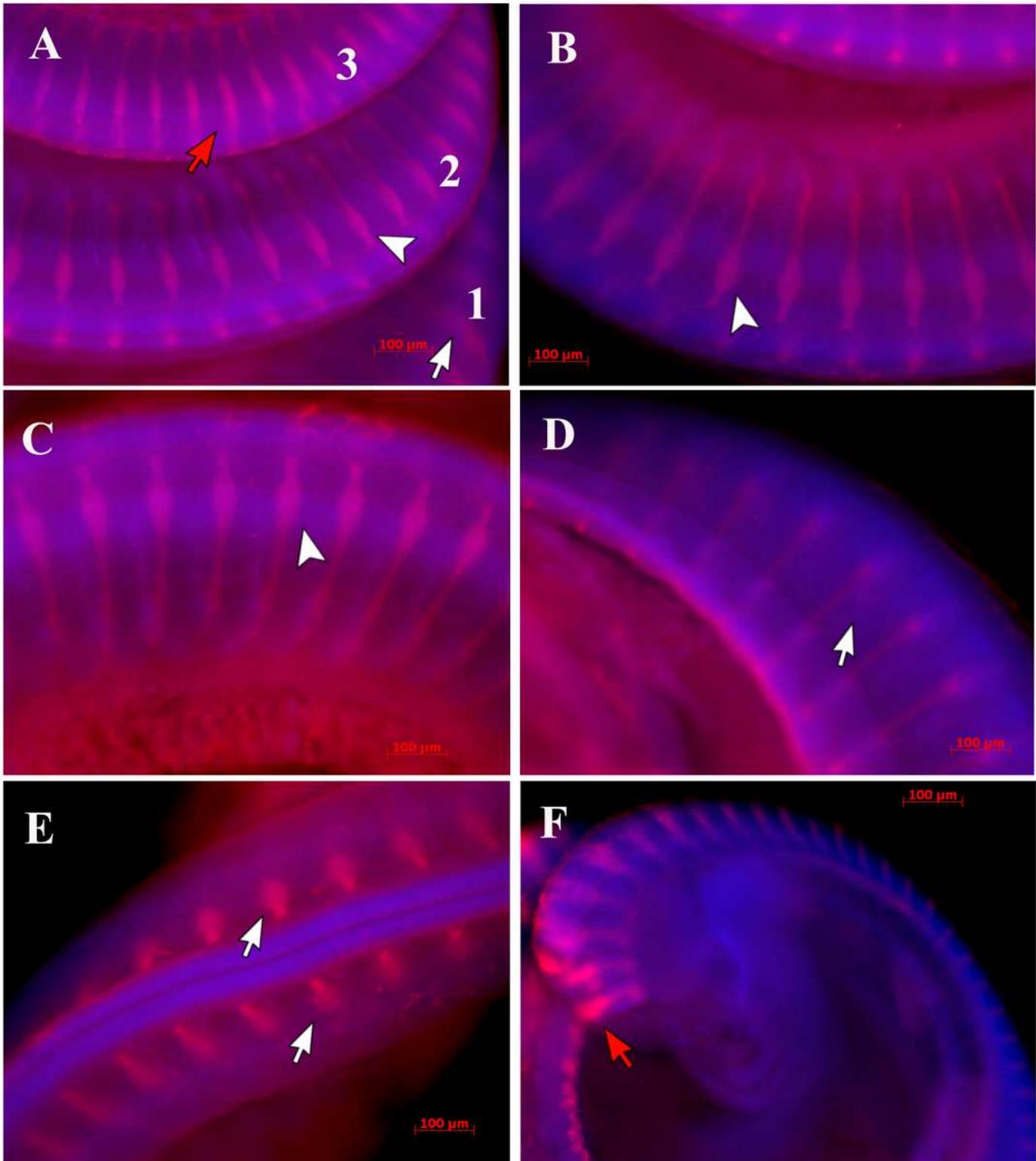


Figure 2

Wholemout of *Naja haje* embryo stage 2/3; 7dpo stained with HNK1 and DAPI. A *Naja haje* embryo (st.3/4) was stained with HNK1 (red) and DAPI (blue). A) Shows the 3 coils of the embryo Coil 1 is the coil closest to the head of the embryo while coil 3 is tail end. BD) Show a more uniform HNK1 staining of migrating TNCC throughout the 3 coils (arrowheads). D) The 1st coil shows less prominent HNK1 stain of TNCC compared to coils 2nd- 3rd (B-C), but we can still observe robust HNK1 the vagal portion of the

embryo (arrow). E) A dorsal view of cobra embryo at this stage shows condensed DRG (arrows). F) Shows TNCC from a rostral (red arrow) to caudal point of view and a reduction in signal can be seen in the caudal-most portion of the embryo.

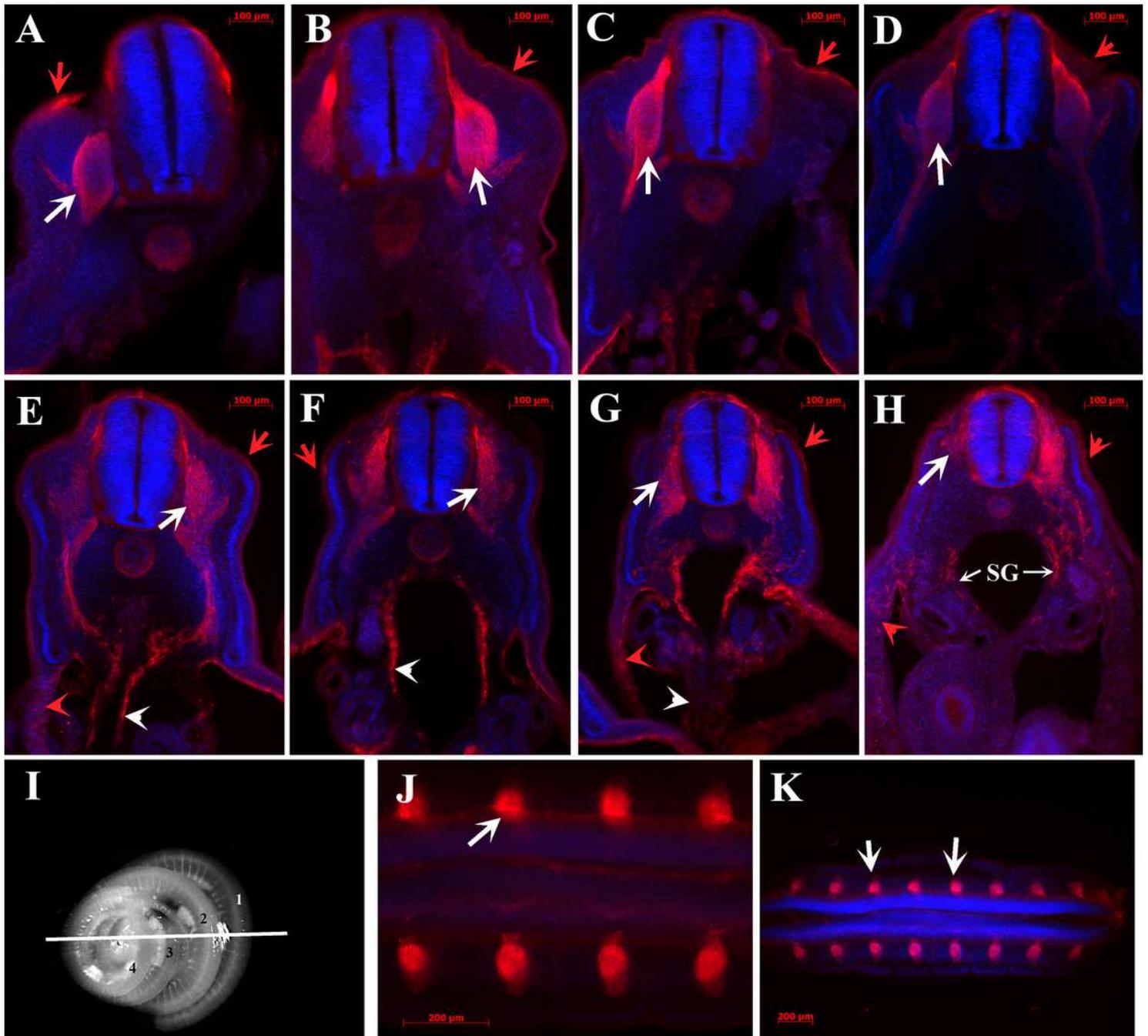


Figure 3

Naja haje embryo stage 2/3; 7dpo sections cut by vibratome and stained with HNK1 and DAPI. Vibratome sections are shown in a rostral to caudal progression (Panels A-H). The DRG's can be seen taking shape the closer at more rostral sections in 1st and 2nd coils (Panels A-C). At more caudal sections DRGs are not clearly condensed (arrows in E-H). I) Shows the wholemount image of the embryo before it was vibratomed and the line shows the level of the sections shown here. J-K) Show two sections from 1st coil with condensed DRGs lateral to the neural tube (arrows). Some of the more caudal sections seem to have

TNCC moving into the gut (white arrowheads in E-H). Red arrows in E, G-H show HNK1 TNCC in lateral mesoderm.

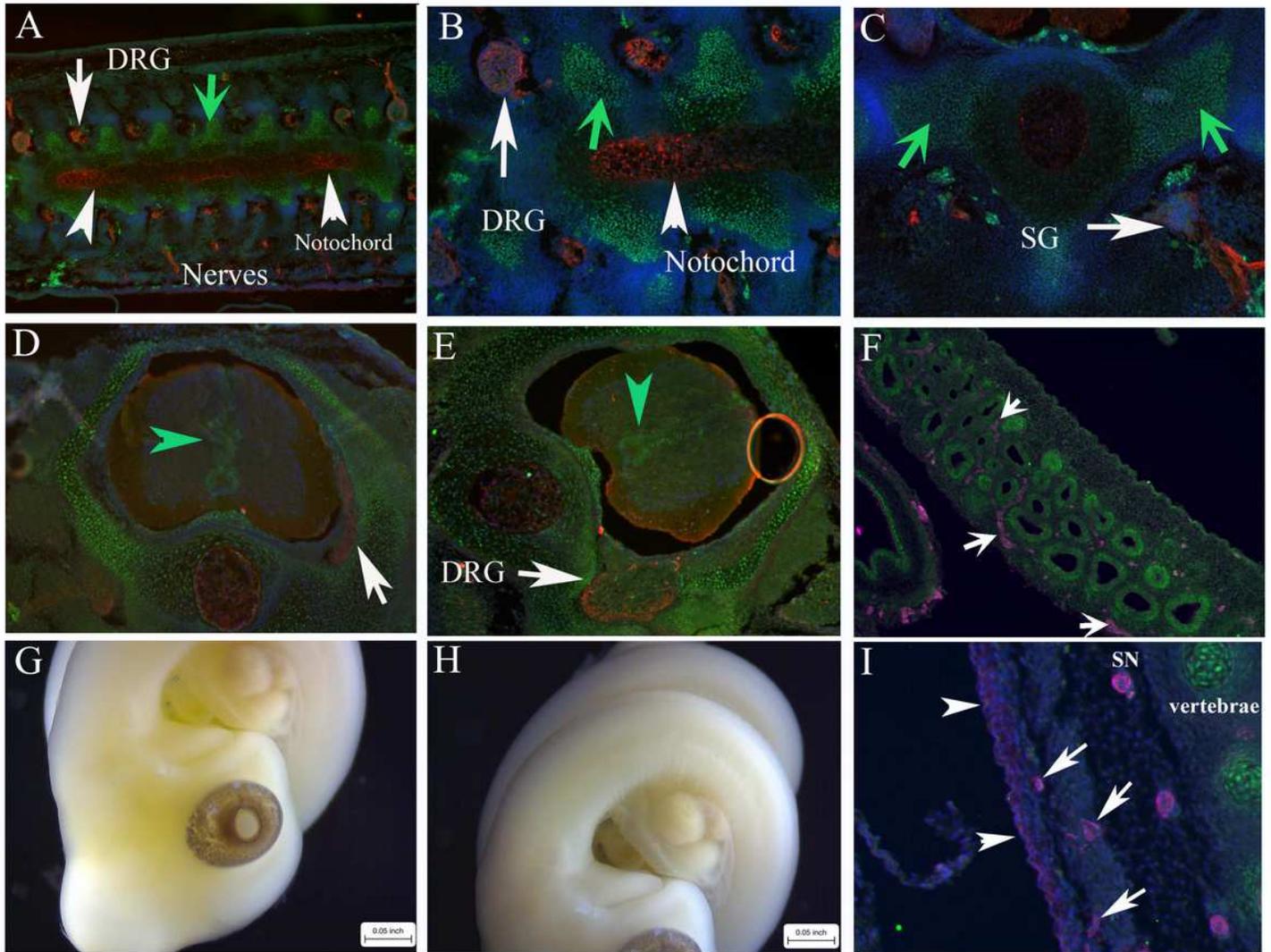


Figure 4

Naja haje embryo stage 4/5 stained with HNK1 and Sox9. Sections through an older embryo (with maxilla and mandible already defined and 3.5 coils) showed a well-developed peripheral nervous system. A-B) Longitudinal sections in second coil showed HNK1 (red) labeling DRGs (green, white arrow), nerves and notochord (white arrowhead), while Sox9 labeled cartilage (green arrow). C) Transverse section of 2nd coil showed HNK1 stained sympathetic ganglia (SG white arrow) and Sox9 stained cartilage (green arrows). D-E) Sections by 3rd coil showed spinal cord stained with HNK1, DRG and dorsal root (white arrow in D). But Sox9 besides staining cartilage, labeled a group of cells in the midline of spinal cord (green arrowhead), likely oligodendrocyte precursors. F) A longitudinal section by the mesonephros showed that these were labeled with Sox9 while HNK1 cells are visible between the tubules (arrows). G-H) Brightfield images of the cobra embryo before sectioning. I) HNK1 labels the external layer of the ectoderm (white arrowheads), while cells and nerves are visible with HNK1 beneath it (white arrows). Spinal nerves and vertebrae are stained with HNK1 and Sox9 respectively.

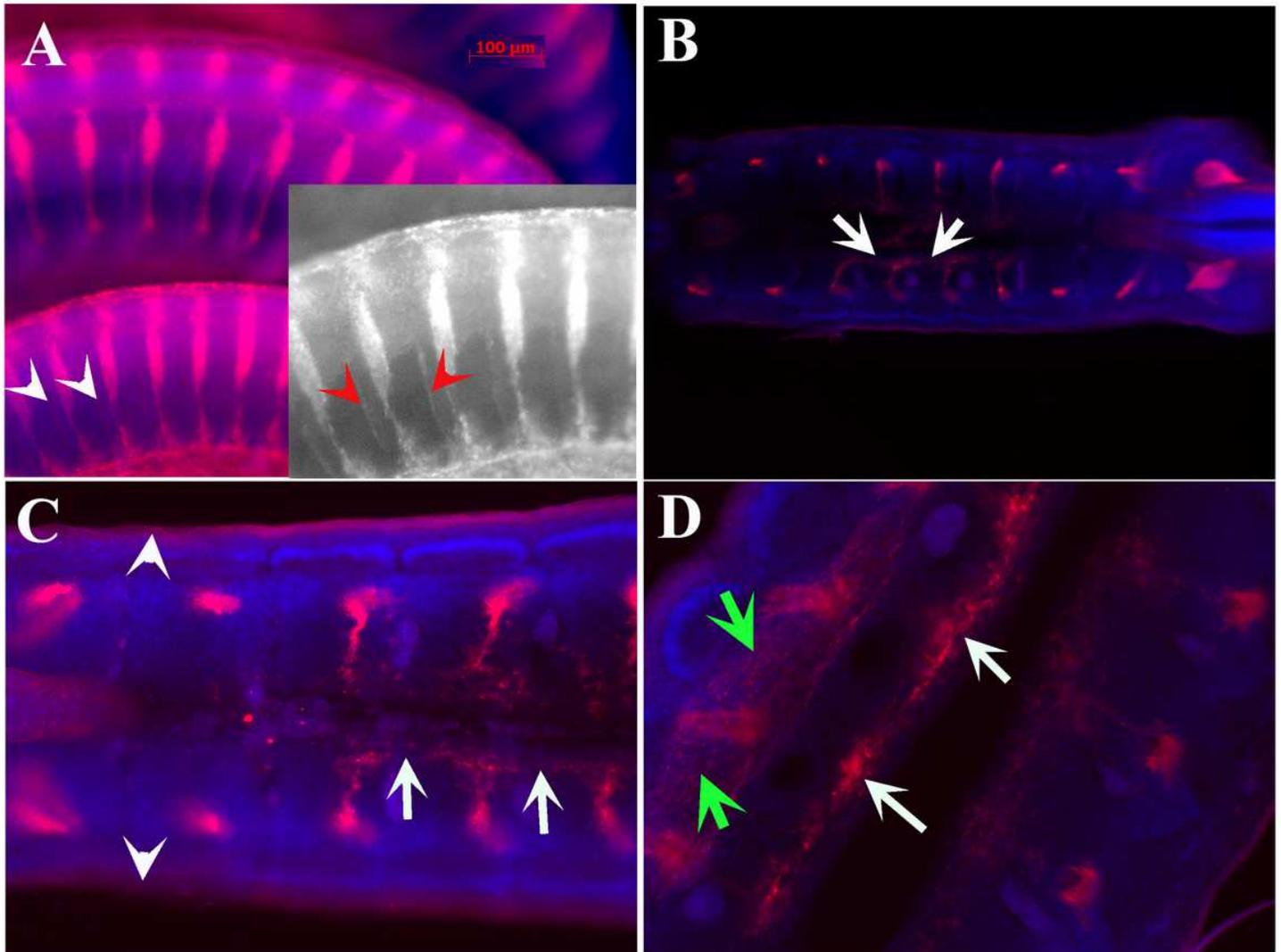


Figure 5

Intersomitic TNCC migration within the Egyptian Cobra *Naja haje*. A) Higher magnifications of Fig.2 embryo (stage 3/4) show the presence of intersomitic HNK1 migrating cells (red arrowheads). B-D) Longitudinal sections of this same embryo along 2nd coil show extensive ventral, next to dorsal aorta intersomitic migration (white arrows). D) Shows extensive staining of ectoderm with HNK1 (white arrowheads) as well as the unique intersomitic HNK1 cells between the rostrally migrating TNCC (green arrows).