EARLY SUBSET OF CEREBELLAR NUCLEI NEURONS DERIVED FROM MESENCEPHALON IN MICE

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Significance Statement

During cerebellar development two germinal zones are involved in cerebellar neurogenesis: the rhombic lip and the ventricular zone, which are located in the developing cerebellum itself. Our findings indicate that a subset of cerebellar nuclei neurons have an external origin, the mesencephalon, and they are the earliest born neurons that enter to the developing cerebellum. In this study, we focused on the origin of these cells and traced their migratory pathway from the mesencephalon while crossing the isthmus, followed them when they entered to the developing cerebellum. We also demonstrated their potential role on later born cells during cerebellar development.

ABSTRACT

During cerebellar development, cerebellar nuclei (CN) neurons and Purkinje cells are the earliest born among the different neuronal subtypes. Purkinje cells are the sole output of the cerebellar cortex and project to the CN. The CN represents the main output of the cerebellum, which is generated from the rhombic lip and the ventricular zone. We used immunohistochemistry, embryonic cultures, dye tracers and *in situ* hybridization to examine the origin of a new subset of CN neurons from the mesencephalon during early cerebellar development. Our results show that a subset of CN neurons, which are immunopositive for α-synuclein (SNCA) and *Otx2*, originate from the mesencephalon and cross the isthmus toward the rostral end of the nuclear transitory zone. Double immunostaining of the SNCA with *Otx2* or p75 neurotrophin receptor (p75ntr) indicates that these cells are derived from neural crest cells. We also showed that this population of neurons with nerve fibers terminates at the subpial surface of putative lobules VI/VII. The $SNCA^{+}$/$Otx2^{+}$/$p75^{+}$ cells, which divide the cerebellar primordium into rosterodorsal and caudoventral compartments, show increased cleaved caspase-3 activation, which suggests temporary presence of these cells due to apoptosis. These results strongly suggest that early CN neurons originate from the mesencephalic neural crest population and cross the isthmus to contribute as a subset of the CN. Their temporary presence in the nuclear transitory zone suggests that these neurons/fibers play a regulatory role as a signaling center to attract early afferent pioneer axons and provide neuronal migratory pathway during early cerebellar development.

INTRODUCTION

The cerebellum is involved in a variety of brain functions including motor and non-motor functions (1, 2). Cerebellar nuclei (CN) are the main structures responsible for cerebellar output channels (3). CN plays a pivotal role in integrating signals, information processing, and cognition (4, 5). During the embryonic stage, CN and Purkinje cells (Pcs) are the only neuronal populations that exist in early cerebellar development (3, 6, 7). In the mouse, the cerebellar primordium emerges at approximately embryonic day (E) E7–E8 as a neuroepithelial swelling on the rostral lip of the fourth ventricle, which is part of the alar plate of the metencephalon (rhombomere-1) (7-10). The cerebellar primordium contains two distinct germinal zones including the ventrally-located ventricular zone (VZ) and dorsally-located rhombic lip (11).

It has been suggested that both glutamatergic and GABAergic CN neurons originate from the cerebellar VZ (3). Further studies highlighted the involvement of the rhombic lip as an origin for glutamatergic CN neurons (between E9-E12) (10-12). To investigate the origin of CN during the cerebellar embryonic stages, our data suggest that the mesencephalon may be a novel origin of newly characterized neurons that contribute to CN formation and development. In this context, the earliest neuronal outgrowth during central nervous system (CNS) development occurs in E9 in the mesencephalic nucleus of the trigeminal nerve (MesV) (13, 14). MesV are considered to be the large sensory neurons and are derived from the neural crest cell (NCC) population (15, 16). At the embryonic stage, NCCs arise from the transient neural crest that initially generates neural crest stem cells, in which migrating cells express the early neural crest marker, p75 neurotrophin receptor (p75ntr; e.g. (17, 18)). Neuronal proliferation/differentiation is regulated by p75ntrs that are mainly expressed during early development (19-21). In addition, previous studies suggest that the orthodenticle homeobox 2 (*Otx2*) plays an essential role in the development of the mesencephalic neural crest-derived neurons and also the cells of the rhombic lip choroid plexus (22-24). The mesencephalic NCCs express *Otx2* during migratory phase (23).

The LIM homeobox transcription factor 1, alpha (*Lmx1a*), is an important regulator of CN neurogenesis which originate from the rhombic lip. It is also expressed in the nuclear transitory zone (NTZ) and is considered to be a marker for majority of rhombic lip-derived CN neurons beside subset of the *Lmx1a* positive cells that do not originate from the rhombic lip migratory stream (25, 26). In this study, we hypothesized that a subset of the CN neurons originate from mesencephalic NCC and migrate caudally toward the NTZ during early cerebellar development. Our findings suggest that the mesencephalon may be a third germinal zone, which is source of the earliest population of the CN neurons and probably present temporarily in the NTZ. These finding are in corroboration with the findings of Nickolus and Bruce (27) which suggest the lineage of Wnt-1 give rise to neural crest and hind brain migration. Additionally, we studied α-synuclein (SNCA; a lipid-associated protein), which is expressed in the somata of CN neurons and axon terminals in the cerebellum, and interacts with proteins such as tau, tubulin, and actin (28-30). We evaluated SCNA because it is considered to be involved in the development of neural stem cells and synaptogenesis in developing neurons (28, 31).

Material and Methods

Animal maintenance

All animal procedures were performed in accordance with institutional regulations and the *Guide to the Care and Use of Experimental Animals* from the Canadian Council for Animal Care. In this study, we used embryos from 36 CD1 timed-pregnant mice at E9 to E18, three CD1 mice at postnatal day (P) P4, three prostatic acid phosphatase (PAP) mutant timed-pregnant mice at embryonic day (E) E12 (The PAP KO mice were obtained from Dr. Pirkko Vihko, University of Helsinki, Finland). We used PAP KO mice (32, 33), since they do not express SNCA and are valuable experimental tool to understand the role of SNCA in subset of CN neurons.

All timed-pregnant CD1 mice were obtained from the Central Animal Care Service, University of Manitoba. Animals were kept at room temperature and relative humidity (18–20°C, 50–60%) on a light and dark cycle (12:12 h) with free access to food and water. The embryo age was determined from the first appearance of a vaginal plug, considered to be E 0.5. CD1 timed-pregnant mice at E (9, 10, 11, 12, 13, 14, 15, 16 and 18) + 0.5 (N=36) and three PAP mutant timed-pregnant mice at E12 were anesthetized [40% isoflurane, USP (Baxter Co. Mississauga, Ontario, Canada)] and embryos were removed and fixed in 4% paraformaldehyde (PFA) for immunohistochemistry (IHC) or prepared for Western Blotting. Three CD1 mice at P4 were transcardially perfused with PBS for IHC, as described previously (34, 35)

Sections immunohistochemistry

Cryostat sections (20 μm) of PFA 4% fixed samples were utilized for IHC process as explained in our previous studies (34, 35). Antibody dilutions were used as follows: α-synuclein (sc-69977, Santa Cruz) 1:500, p75NTR (8238, Cell Signaling) 1:1000, Lmx1a (AB10533, EMD Millipore Corporation) 1:500, Otx2 (ab114138, abcam) 1:1000, NAA (3A10, Developmental Studies Hybridoma Bank) 1:500, βTubulin (T8328, Sigma-Aldrich) 1:2000, TrkA (ab76291, abcam) 1:50, and Cleaved Caspase 3 (9664, Cell

Signaling) 1:200. Fluorescent detection was performed using antibodies as follows: Streptavidin, Alexa Fluor® 488 conjugate, Alexa Fluor® 568 Goat Anti-Rabbit IgG (H+L), Alexa Fluor 488 Chicken Anti-Mouse IgG (H+L), Alexa Fluor 488 Chicken Anti-Rabbit IgG (H / L), and Alexa Fluor 568 Goat Anti-Mouse IgG (H+L) (S-11223, A-11036, A21200, A21441, A11004 Life Technologies) 1:1000. Detection of peroxidase IHC was also performed as described previously (34-36) using HRP conjugated goat anti-rabbit IgG and goat anti-mouse IgG (H+L) antibodies (EMD Millipore Corporation, 12-348 and AP308P, respectively) 1:500, and developed with DAB (3,3'-diaminobenzidine) solution (Sigma, St. Louis MO, USA).

Whole mount immunohistochemistry

Whole mount IHC was performed on embryos according to Sillitoe and Hawkes (37) with a few modifications. Briefly, PBS containing 0.2% skim milk (Nestlé Foods Inc., North York ON, Canada) plus 0.1% Triton-X 100 (Sigma, St. Louis MO, USA) and 5% dimethyl sulfoxide (DMSO) at 4ºC overnight was used as the blocking solution (PBSMT). After primary and secondary incubation, the immunoreactivity was revealed by DAB, and then the tissue was washed in PBS and stored in PFA for analysis.

Primary dissociated cerebellar culture

Primary cerebellar cultures were prepared from embryonic (E) day 10 CD1 mice, and cells were maintained for varying days *in vitro* (DIV 1, 2, 3, 5, and 8), according to published methods (35). Briefly, the entire cerebellum was removed from each embryo and immediately placed into ice cold Ca^{2+}/Mg^{2+}-free Hank's balance salt solution (HBSS) containing gentamicin (10 µg/ml) and glucose (6 mM). The cerebella were incubated at 34°C for 12 min in HBSS containing 0.1% trypsin. After washing, the cerebella were gently triturated in HBSS containing DNase I (5 U/ml) and 12 mM $MgSO_4$ until the cell

mass was no longer visible. The cells were collected by centrifugation (1,200 rpm, 4°C for 5 min) and re-suspended in seeding medium (1:1 Dulbecco's modified Eagle's medium and F12) supplemented with putrescine (100 μM), sodium selenite (30 nM), L-glutamine (1.4 mM), gentamicin (5 μg/ml), and 10% heat-inactivated fetal bovine serum. The cell suspensions were seeded on poly-L-ornithine coated glass coverslips (12 mm) at a density of 5×10^6 cells/ml, with each coverslip placed into the well of a 24-well plate. After 6–8 h incubation in a CO_2 incubator (100% humidity, 37°C, 5% CO_2), 500 μl of culture medium supplemented with transferrin (200 μg/ml), insulin (20 μg/ml), progesterone (40 nM), and triiodothyronine (0.5 ng/ml) was added to each culture well. After 7 days, half of the medium in each dish was replaced with fresh medium that was additionally supplemented with cytosine arabinoside (4 μM) and bovine serum albumin (100 μg/ml) (35, 38, 39).

Embryonic cultures and DiI labeling of cells within the mesencephalon

Embryonic cultures were prepared from E9 and E10 CD1 timed-pregnant mice, and embryos were maintained for various DIV (4, and 6). Each embryo was removed from the amniotic sac and immediately placed into ice cold Ca^{2+}/Mg^{2+}-free HBSS containing Gentamicin (10 μg/ml) and glucose (6 mM). Embryos were placed into 24-well plates in culture medium plus 10% fetal bovine serum and incubated in a CO_2 incubator (100% humidity, 37°C, 5% CO_2) (38). Embryos were monitored every 6 h to evaluate the heart beat during incubation as a survival sign. On the desired day, each well was fixed with 4% PFA and prepared for whole mount IHC.

For neuronal tracing and labeling, we used the FAST DiI crystal (FAST DiI™ solid; DiIΔ9,12-C18(3), CBS (1,1'-Dilinoleyl-3,3,3',3'-Tetramethylindocarbocyanine, 4-Chlorobenzenesulfonate, D7756, Fisher Scientific). Briefly, FAST DiI was inserted to the mesencephalon at E9 using a sharp-ended needle. After insertion of FAST Dil, images were captured by stereomicroscope to monitor the location of DiI at day *in vitro* 0 (DIV 0). After placing the embryos into 24-well plates in culture medium, embryos were

monitored every 6 hours and fixed with 4% PFA on the desired day, then whole mount IHC with NAA was performed to visualize neural fibers growth and followed by sectioning and imaging of the DiI positive cells in mesencephalon and cerebellar primordium.

In Situ Hybridization (ISH)

All of the ISH experiments were carried out on the E12 CD1 mice using RNAscope ACD HybEZ™ II Hybridization System and RNAscope® Multiplex Fluorscent Reagent Kit v2 (Advanced Cell Diagnostics, Hayward, CA, USA). Briefly, embryos were fixed in 10% (vol/vol) neutral buffered formalin at room temperature for 24 h, dehydrated, and embedded in paraffin. Tissue sections cut at 10 μm thickness were processed for RNA *in situ* detection according to the manufacturer's user manual. Sequences of the probes used in the study are as follows: Mm-Fgf8 (313411, ACD), Mm-Wnt1-C2 (401091-C2, ACD), Mm-Otx2-C3 (444381, ACD). Flourophores (TSA® Plus, Perkin Elmers, Waltham, MA, USA)) used in the study are as follows: Fluorescein (NEL741E001KT), Cyanine 3 (NEL744E001KT), and Cyanine 5 (NEL745E001KT).

Western Blotting Analyses

Equal amount of proteins were separated by SDS/PAGE in 10–15% precast gels (Bio-Rad, Hercules, CA, USA) and transferred onto the PVDF-membrane. For the Western blot analysis, membranes were blocked in 5% nonfat dry milk (NFDM) in TBS containing 0.02% Tween 20 (TBST) and then incubated overnight at 4°C with primary antibodies as follows: α-synuclein (sc-69977, Santa Cruz) 1:2000, p75NTR (8238, Cell Signaling) 1:1000, Caspase 3 (9665, Cell Signaling)1:1000, and Cleaved Caspase 3 (9664, Cell Signaling) 1:1000. Secondary antibodies as follows: HRP conjugated goat anti-mouse IgG (AP308P, Millipore) 1:6000 and HRP conjugated goat anti-rabbit IgG (12-348, Millipore)

1:6000. Binding was assessed using the Enhanced Chemiluminescence (ECL) protocol on Scientific Imaging Film.

Imaging and figure preparation

For bright field microscopy, images were captured using Zeiss Axio Imager M2 microscope (Zeiss, Toronto, ON, Canada). Images were then analyzed with a Zeiss Microscope Software (Zen Image Analyses software) (Zeiss, Toronto, ON, Canada). For fluorescence microscopy of the entire cerebellum sections, a Zeiss Lumar V12 Fluorescence stereomicroscope (Zeiss, Toronto, ON, Canada) equipped with a camera was used to capture the images. Images were then analyzed using Zen software. For high magnification fluorescence microscopy, a Ziess Z1 and Z2 Imager and a Zeiss LSM 700 confocal microscope (Zeiss, Toronto, ON, Canada) equipped with camera and Zen software were used to capture and analyze images. Images were cropped, corrected for brightness and contrast, and assembled into montages using Adobe Photoshop CS5 Version 12.

RESULTS

The early subset of CN neurons at the rostral end of the cerebellar primordium are derived from the mesencephalon

In mouse embryos, at the rostral end of the cerebellar primordium a few cells are immunopositive for neuronal marker such as neurofilament-associated antigen (NAA) 3A10 (40) at the early stage of E9 (Fig. 1A-D). To explore whether these cells cross the isthmus, we used anti-alpha-synuclein (SNCA) antibody, which is expressed in subset of CN neurons (28). In a sagittal section of the E9 cerebellar primordium, we found that SNCA$^+$ neurons run from the dorsal mesencephalon, cross the isthmus, and pass in through the rostral end of the NTZ (Fig. 1E-F). To determine the position of the SNCA$^+$ cells in the NTZ compared to the rhombic lip-derived *Lmx1a*$^+$ CN neurons (E10-12) (11), double immunocytochemistry of SNCA and *Lmx1a* was performed on sagittal sections of E12 cerebellar primordium (Fig. 2 A-D, medial section and Fig. E-H, lateral section). This shows that an *Lmx1a*$^+$ population of CN neurons, which extends from the rhombic lip, flanks SNCA$^+$ neurons in the NTZ that is continues rostrally to the mesencephalon (Fig. 2A, E). The majority of neurons are not SNCA+/*Lmx1a*$^+$ but some of the neurons are co-labeled, and the latter may be because the cells are overlapped (Fig. 2 D, H).

To explore the possibility that the SNCA$^+$ cells originate from the mesencephalon, *Otx2* IHC was performed during early cerebellar development. *Otx2* is highly expressed in the mesencephalon and its caudal limit is the boundary with the metencephalon (i.e. the isthmus) (22). However, IHC at the sagittal section of E12 cerebellum showed that a subset of *Otx2*$^+$ cells from the mesencephalon continue caudally, cross the isthmus and end in the rostral region of cerebellar primordium (Fig. 3 A-B, medial) and Fig 3 C-D, lateral). To understand whether the *Otx2*$^+$ cells that continue to the NTZ are SNCA$^+$, double IHC with *Otx2* and SNCA was performed in E12 sagittal sections. *Otx2* is highly expressed in the mesencephalon

(Fig. 4A, B), and was co-expressed with $SNCA^+$ cells in the NTZ (Fig. 4C-E). Primary dissociation of cerebellar culture at E10 and DIV 4 showed that $SNCA^+$ cells were co-expressed with *Otx2* (Fig. 4 F–H).

To determine whether mesencephalic-derived early CN neuron express*Otx2*, *Wnt1*, and *Fgf8* in cerebellar primordium at E12, we employed highly-sensitive RNAscope ISH, which allowed us to simultaneously detect three probes. *Otx2* mRNA signal is highly expressed in the mesencephalon and extend a tail caudally cross the isthmus to the rostral cerebellar primordium in NTZ (Fig 5 D). *Wnt1* mRNA signal is high in the mesencephalon and isthmus similar to $Otx2^+$ cells, extend to the rostral cerebellar primordium in NTZ (Fig 5. E). *Fgf8* mRNA signal is present in few scattered cells in rostral end of the cerebellar primordium (Fig 5. F). $Otx2^+$ cells at NTZ are co-expressed with $Wnt1^+$ cells (Fig5. A, B and G).

The results of IHC and ISH experiment suggest that the subset of CN neurons are possibly derived from the mesencephalon. To confirm these findings, we used FAST Dil as a neuronal tracer to further investigate mesencephalic derived CN cell migration. We applied FAST DiI with a sharp-ended needle in dorsum of caudal mesencephalon (Fig. 6 A, a). After 4 days *in vitro*, mesencephalic-derived cells which are stained with DiI clearly present and migrate in both rostrally (Fig. 6B) and caudally (Fig. 6C) directions. Sections from the mesencephalon and cerebellar primordium shows that mesencephalic derived cells are present in the rostral of the cerebellar primordium in putative NTZ in medial (Fig. 6 D-F) and lateral (Fig. 6 G-I) sections. To expose the earliest cell population in mesencephalon to DiI and avoid of unwanted cell staining due to long exposure to DiI, we focused on early time point of the cerebellar development, i.e. at E9. It is reported that the earliest neuronal population in CNS are present in mesencephalon and project caudally (41). To determine whether early generation of mesencephalic cells migrate to the cerebellar primordium, the DiI exposure time to mesencephalic cells limited for only 24 hours (Fig. 7 A,a) and then the inserted DiI removed (Fig. 7 B, E). To our surprise, almost all DiI+ cells migrated caudally toward the cerebellar primordium, not rostrally (Fig. 7B, C, D, E). The DiI staining

is clear and strong in some cells (Fig. 7 D, d and arrowhead in G'), although the DiI+ red signal is seen in other cells in the rostral end of the cerebellar primordium, but is weak and seems fading (Fig. 7 E-G").

Early CN neuron originate from mesencephalic neural crest

The mesencephalic neural crest cells during the migratory phase express *Otx2* gene (23). At the embryonic stage, neural crest stem cells (NCSCs) arise from the transient neural crest and create tissues that initially generate neural crest stem cells, in which migrating cells express p75ntr, the early neural crest marker (19). Neuronal proliferation/differentiation and the neuronal fiber formation are regulated by p75ntr, which is mainly expressed during early development (20, 21). The p75ntr (a typical neural crest cell marker) (17, 18) was used to test for $SNCA^{+}/Otx2^{+}$ cells that arise probably from the neural crest population.

To determine whether $SNCA^{+}/Otx2^{+}$ neurons are p75ntr immunopositive, double immunostaining with SNCA was performed. Section IHC revealed that $SNCA^{+}$ cells in the NTZ were co-labeled with p75ntr that is localized in the cell membrane (Fig. 8A–C, H). This data was further confirmed by Western blot analysis of SNCA and p75ntr expression during different embryonic days, E11, E13 and E15 (Fig. 8h). In addition, in primary dissociated cerebellar culture at E10, DIV 3 showed that p75ntr is expressed with $SNCA^{+}$ neuronal cell membrane (with punctate appearance, Fig. 8 D–G (21)). P75ntr and TrkA (which also labels neural crest-derived neurons) (42-45) are two distinct nerve growth factor receptors that probably form a ternary TrkA/NGF/p75 complex (46). To determine whether TrkA is expressed in a subset of neurons in the NTZ, we have performed double immunostaining with NAA and showed TrkA and NAA co-expression in the NTZ (Fig. 8 I–K).

Immunostaining with NAA 3A10 showed that a set of the neurons and neuronal processes are present in the NTZ in the cerebellar primordium core at E12 (Fig. 9A). To determine whether the NAA $3A10^{+}$ combination of neurons and neuronal processes at the core of cerebellar primordium express

p75ntr, double immunostaining was performed (Fig. 9A–C, H). The results showed that the $NAA^+/p75ntr^+$ combination of neurons and neuronal processes from the isthmus continue to the NTZ and terminate on the subpial surface at the midpoint of the rostrocaudal cerebellar primordium (Fig. 9H). In dissociated embryonic cerebellar culture at E10, DIV 21 showed that p75ntr immunoreactivity is localized in the membrane of NAA^+ neuronal somata and process (Fig. 9 D–G).

Double IHC of p75ntr with SNCA at E10 showed that expression is mostly localized in neurons at the caudal end (Fig. 10A–D), while at E12, expression is also localized in the neurons at the rostral end of the NTZ (Fig. 10 E–H). $P75ntr^+/SNCA^+$ neurons and neuronal processes combine at the cerebellar primordium core and terminate on the subapical surface at the rostrocaudal midway point of the cerebellar primordium, probably at putative lobules VI/VII (Fig. 10 A–H).

Transient expression of SNCA in a subset of CN neurons

To understand the fate of SNCA and SNCA+ cells in a subset of CN neurons, the following experiments were performed. First, we determined that the pattern of SNCA expression in the rostral cerebellar primordium was prominent in the CN from E9 to E13(Figs 1 and 2). At E14, SNCA expression was weak in neuronal cell body (Fig. 11A, B, b). At E15/16, SNCA expression disappeared from the CN neuronal cell bodies (28) (data not shown). Around the perinatal cerebellum, SNCA expression was localized to the axonal terminal field and there was no SNCA expression in any cell body on the cerebellar cortex and CN (Fig. 11 C–E). To understand the role of SNCA in the mesencephalic derived CN neurons, we used PAP mutant mouse cerebellum, in which the expression of α-synuclein (SNCA) is absent. In our study, using RT-PCR for PAP-null mice, genomic DNA showed that SNCA is not present in this mouse (supplementary 12 A). IHC in the adult control cerebellum shows SNCA expression in the axonal terminal, while SNCA is not expressed in the PAP-null mouse (supplementary 12 B). The lack of SNCA expression in PAP-null cerebellum has also been confirmed by Western Blot (data not shown). Thus,

PAP-null mouse embryo is a promising model to understand the role of SNCA in this subset of CN neurons in the NTZ during early cerebellar development. Double IHC with SNCA and p75ntr at E12 showed that the p75ntr^{+} cells are present in NTZ, but there was no SNCA expression compared with wild type (Fig. 12 A-F). PAP-null mouse behavior and motor activity was monitored for about 15 months, and no obvious motor disability was observed. However, PAP-null mice seemed to be more active compared with the wild type mice even at older ages.

To further our understanding of the SNCA expression in early cerebellar development, primary dissociated cerebellar culture at E12, DIV 5 was double immunostained with β-tubulin. This experiment shows localization of SNCA in cytoplasm (Fig. 8G and 13E), and SNCA can also be localized in the nucleus (Fig. 13 A–D). Embryonic cerebellar culture from mouse embryo at E10 revealed that SNCA expression is localized in both the cytoplasm (arrow) and nucleus (arrow head) at DIV 6 (Fig. 13 E–G); however, at DIV 13, SNCA seems to be aggregated in cells or cell clusters (Fig. 13 H–J). SNCA aggregation is a major cause of neurodegeneration (47), and therefore it may indicate that SNCA^{+} cells in the NTZ undergo the cell death. To explore whether SNCA^{+} cells undergo programmed cell death, cleaved caspase-3 IHC was performed and the results showed that some SNCA^{+} cells in the NTZ express cleaved caspase-3 (Fig. 14 A-D). Western blot analysis revealed activation of caspase 3 in embryonic cerebellum (Fig. 14 A). In early embryonic cerebellar sections, cleaved caspase-3 was present in a combination of neurons and neuronal fibers, and direct toward the subpial surfaces at the mid-point between the rostral and caudal cerebellar primordium (Figs. 10 E, and 14 A–D). Cleaved caspase-3 activity is also high in the ophthalmic component of the trigeminal ganglion, a source of early afferent to cerebellar primordium (Fig. 14 F–G). Trigeminal ganglion is outlined in whole mount immunohistochemistry with NAA (Fig. 14E).

DISCUSSION

In this study, we investigated the mesencephalic-derived CN neurons during early cerebellar development. We showed that a novel subset of CN neurons (SNCA$^+$/*Otx2*$^+$ neurons) run from the dorsal mesencephalon and cross the isthmus to the NTZ as early as E9. This suggests the presence of a new germinal zone during cerebellar neurogenesis. Further, these cells are *Otx2*$^+$/p75ntr$^+$/Wnt1$^+$/TrkA$^+$, suggesting that this subset of CN neurons is probably derived from the mesencephalic neural crest population. This novel subset of CN neurons are accompanied by the nerve fibers that express activated caspase-3, restricted within the cerebellar primordium core. Our results indicate that this subset of the CN neurons/fibers are temporarily present at the cerebellar primordium core and terminate at the midway point of the rostrocaudal subpial surface. This suggests that the mesencephalic derived SNCA$^+$/*Otx2*$^+$/p75ntr$^+$ combination of neurons/fibers probably plays a role as a transient signaling center.

Early subset of CN neurons originate from the mesencephalon

Recent genetic fate mapping studies suggested that most CN projection neurons may arise from the rhombic lip (11, 48, 49). The transcription factor expression patterns suggest that CN neurons migrate from the rhombic lip to the NTZ through a subpial stream pathway while sequentially expressing the genes *Pax6*, *Tbr2*, and *Tbr1* (7, 11).

The origin of the SNCA$^+$ neurons is currently not clear, but the rostral continuity of these neurons from the NTZ indicates that they may arise from the mesencephalon. *Tbr1*$^+$/*Lmx1a*$^+$ neurons are born in the rhombic lip at E9, but they do not reach the NTZ until around E10 (11, 50). Our study showed that SNCA$^+$ cells are a group of differentiating neurons (NAA$^+$) present in NTZ at E9, before the arrival of any neurons that originate in the rhombic lip. Although, most SNCA$^+$ neurons are not *Lmx1a*$^+$, some of the neurons are co-labeled with SNCA. This suggests that in the early stages of CN neurogenesis, the pattern of protein expression in SNCA$^+$ neurons is changing, possibly with SNCA being down-regulated

and *Lmx1a*$^+$ being up-regulated, or this may not occur at all (Fig. 2). The NTZ is an intercalated area of the SNCA$^+$/*Lmx1a*$^+$ cells, and it shows the continuation of SNCA$^+$ to the rostral end and *Lmx1a*$^+$ to the caudal end as a source of origin.

To further study this hypothesis, we investigated *Otx2* expression in this subset of neurons in the developing cerebellum. *Otx2* has been shown to be involved in prosencephalon and mesencephalon establishment, but not the rhombencephlon (51, 52). *Otx2* is required for the development of the forebrain and midbrain, while *Gbx2* is necessary for development of the anterior hindbrain (53, 54). Our results indicate that the SNCA$^+$ cells in the NTZ express *Otx2*. In addition, *Wnt1* which is express in mesencephalon (55) is co-expressed with *Otx2*$^+$ cells in rostral cerebellar primordium, but not *Fgf8*. Considering that mesencephalic cells are *Otx2*$^+$ (23), we suggest that SNCA$^+$ and *Otx2*$^+$ cells in cerebellar primordium originate from the mesencephalon. Using embryonic culture with inserted DiI, we clearly showed that mesencephalic derived cells through the rostral end migrate to the cerebellar primordium. This strongly suggests that the caudal mesencephalon may play a role as a third germinal zone, which forms a subpopulation of CN neurons that likely predates all neurogenesis in the cerebellar primordium.

SNCA$^+$*/Otx2*$^+$ *cells with p75ntr/NAA reveal a combination of neurons and neuronal fibers at the core of cerebellar primordium*

The neuronal precursor in the mesencephalon arises from neuroepithelium and neural crest cell population (13, 15, 16). In the mesencephalon, the mesencephalic nucleus of the trigeminal nerve is the large sensory neurons which are derived from the neural crest (16, 56, 57). They are the earliest neuronal outgrowths that have been detected in the central nervous system, and are the first differentiated neurons in the mouse brain at E9 (13, 16). It is also suggested that *Otx2* is expressed in migrating mesencephalic neural crest cells (23). We showed that SNCA$^+$/*Otx2*$^+$ cells are *Wnt1*$^+$ that is express in precursors of the neural crest and substantially contribute to the CN (58). In addition, we showed that SNCA$^+$/*Otx2*$^+$ cells

are p75ntr$^+$ and TrkA$^+$, both are the nerve growth factor (NGF) receptor expressed in neural crest cells (18, 42, 45, 46). Therefore, this suggests that SNCA$^+$/*Otx2*$^+$/p75ntr$^+$/TrkA$^+$ neurons in the NTZ arise from the mesencephalic nucleus of the trigeminal nerve. The neural crest cells are a highly migratory multipotent cell population that arises from the neural fold and gives rise to a wide variety of cell types (e.g. (59-61)). A neural crest-derived subpopulation in the CNS represents a great research potential because neural crest-derived cells may have the capacity for neurogenesis and regeneration, e.g. in the olfactory system (62, 63).

Transient expression of SNCA in mesencephalic-derived CN neurons

During cerebellar development, the prominent SNCA expression in CN neuron somata is transiently initiated, and at around E15–16, its expression in the developing cerebellum may diffused into neuronal fibers (Fig. 9). This dynamic expression pattern is in agreement with other studies on SNCA expression patterns in embryos (28). In our study, we did not detect SNCA expression in any neuronal somata in the cerebellar cortex and CN during perinatal development. It has been suggested that the SNCA may be involved in neuronal migration, neural cell fate and differentiation (64). However, our study on the PAP/SNCA-null mouse showed that the mesencephalic-derived cells migrate to the NTZ, which indicates that a lack of SNCA is not involved in the migration. Therefore, the cytoarchitecture and function of the cerebellum was not affected despite the absence of SNCA, and there may be a redundancy using other molecules to compensate for the loss of SNCA function.

The nuclear localization of SNCA was also reported in several studies in both physiological and pathological situations (65, 66). SNCA overexpression and its aggregated form have been shown to be involved in neurotoxicity (28, 66, 67). Our results demonstrate SNCA aggregation in the embryonic cerebellar culture of an E10 cerebellar primordium after 13 days *in vitro* (DIV 13), but not at DIV 6. The SNCA$^+$/*Otx2*$^+$/p75ntr$^+$ combination of neurons/fibers at the core of the cerebellar primordium showed an

unexpectedly activated CC3 that began from the mesencephalon and terminated at the midpoint of the rostrocaudal cerebellar primordium.

The transient presence of $SNCA^+$ cells in the core of the early cerebellar primordium suggests that they have a temporary regulatory role between the rostrodorsal and caudoventral domains in the developing cerebellum. The neuronal fibers probably originate from early CN neurons or from pioneer axons that originate from the trigeminal nerve with or without synapses containing $SNCA^+/Otx2^+$ cells that terminate on the subpial surface at the point that rhombic lip-derived $Lmx1a^+$ CN changes direction from tangential migration to approximately radial migration. Therefore, it may provide a substrate pathway that directs the $Lmx1^+$ cells to migrate to their destination in the NTZ. Given that rhombic lip-derived $Lmx1a^+$ neuron migration to the NTZ is paved by pioneer neural fibers, they are expected to disappear soon after migration because CC3 is highly expressed and these $CC3^+$ fibers may play a part in axonal guidance and neuronal migration during early cerebellar development. However, this set of pioneer neural fibers persists and can be traced up to the perinatal period of cerebellar development. Thus, this set of pioneer neural fibers may have dual functions, and its second role probably points to the midway position of subpial termination and indicates the putative lobule VI/VII at the paramedian sulcus. The significance of this termination around the perinatal period at the paramedian sulcus probably causes an area with no cerebellar cortex, known as an acortical area, in some adult cerebelli, such as in the bat (68) and the naked mole rat (69). However, this does not happen for all species (e.g. mouse (40)). It is not clear whether the presence of this set of fibers until around the perinatal period is involved in acortical area formation in paramedial sulcus in the cerebellum. Finally, the prominent presence of early combinations of neurons/fibers may indicate an organizing center at the cerebellar primordium core, and continuation neuronal fibers could be a landmark for anterior and posterior development.

Conclusion

Our study suggests that a subset of CN neurons that is SNCA^{+}/*Otx2*$^{+}$/p75ntr^{+} originates from the mesencephalic neural crest, which is a possible new germinal zone for cerebellar development. We showed that this subset of the CN neurons accompanies nerve fibers that are temporarily present at the core cerebellar primordium and terminates at the midway of the rostrocaudal subpial surface. This suggests that they play a role as a secondary organizer that may act as a transient signaling center. This center probably directs rhombic-derived cells to the NTZ and also organizes the cerebellum into anterior and posterior divisions at the level of lobule VI/VII. We are only beginning to understand the role of mesencephalic-derived CN neurons that accompany the neuronal fibers at the core of the cerebellar primordium, and further studies are required to determine the precise molecular mechanism and functional significance of this putative transient center.

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FIGURES

Fig. 1. Cerebellar primordium immunostained with NAA and SNCA shows that a subset of neurons is present at the rostral end of cerebellum at E9

A. Dorsal view of the schematic illustration of the cerebellar primordium, mesencephalon, isthmus, and 4th ventricle. The red line shows the sagittal plane about which the section shown in B–D was taken.

B-D. Sagittal section through the cerebellar primordium at early stage E9. Immunoperoxidase-stained with NAA 3A10 shows the presence of neurons in the cerebellar primordium that crosses the isthmus (i) and continues to the mesencephalon. **C.** A higher magnification of **B**. **D.** Differentiated neurons at E9 are visible; a higher magnification is shown in the inset, **d**.

E–F. Sagittal section through the cerebellar primordium at late E9. Immunofluorescence staining of SNCA shows $SNCA^+$ (green) CN neurons in the mesencephalon, at the isthmus (i) and in the rostral part of cerebellar primordium (cb). **F.** A higher magnification of **E**.

Abbreviations: 4thv, 4th ventricle; cb, cerebellum; i, isthmus; m, mesencephalon; r, rostral; c, caudal; d, dorsal; v, ventral

Scale bar, 100 µm in B; 50 µm in C, E; and 20 µm in D

Fig. 2. Sagittal section through the cerebellar primordium at E12 double immunofluorescence stained with SNCA and *Lmx1a* at medial (A–D) and lateral (E–H) sections

A–G. SNCA (green, A and E) and *Lmx1a* (red, B and F) immunopositive cells are located at a CN neuron temporary site called the NTZ. The $SNCA^+$ cells continue to the mesencephalon and $Lmx1a^+$ cells continue to the rhombic lip. Merged images show that the $SNCA^+$ form a population of CN neurons distinct from

the rhombic lip-derived cells (*Lmx1a*$^{+}$) in NTZ (C and G). D and H show a higher magnification of C and G, respectively.

Abbreviations: 4thV, 4th ventricle; cb, cerebellum; i, isthmus; m, mesencephalon; rl, rhombic lip; r, rostral; c, caudal; d, dorsal; v, ventral

Scale bar, 100 µm

Fig. 3. Sagittal section through the cerebellar primordium at E 12, peroxidase immunostained by *Otx2*

A–D. Sagittal section through medial (A) and lateral (C) cerebellar primordium show high *Otx2* immunoreactivity at the mesencephalon and a few *Otx2*$^{+}$ cells cross the isthmus and position at the rostral part of cerebellar primordium at the NTZ. The boxed regions in **A** (a) and **C** (b) are shown at higher magnification in **B** and **D**, respectively.

Abbreviations: 4thv, 4th ventricle; i, isthmus; m, mesencephalon; rl, rhombic lip

Scale bar, 200 µm in A, C; 50 µm in D, B

Fig. 4. Sagittal section through the cerebellar primordium at E12 double immunofluorescence stained with SNCA and *Otx2* in section and primary cerebellar culture

A–E. Double immunostaining of SNCA (green) and *Otx2* (red) on the sagittal section of the cerebellar primordium at E12. **A**. High *Otx2* immunoreactivity in the mesencephalon. SNCA^{+} cells in the mesencephalon accompany *Otx2* $^{+}$ cells cross the isthmus (i) and enter the NTZ. **B**. higher magnification of **A**. **C-E**. Immunostaining with SNCA (green, **C**) and *Otx2* (red, **D**) and merged (**E**) shows co-expression in the NTZ.

F–H. Primary dissociated cerebellar culture obtained at E10, DIV 4 shows co-expression of $SNCA^{+}$ cells with *Otx2*.

Abbreviations: 4thv, 4th ventricle; CB, cerebellum; i, isthmus; m, mesencephalon; rl, rhombic lip; NTZ, nuclear transitory zone

Scale bar, 200 µm in A; 50 µm in B, F, G, H; 20 µm in C, D, E

Fig. 5. Expression patterns of *Otx2*, *Wnt1*, and *Fgf8* ligands at E12 mouse cerebellum evaluated by RNA *in situ* hybridization (RNAscope). *In situ* hybridization of all ligands (*Otx2* red, *Wnt1* yellow, and *Fgf8* green) on embryo sections from CD1 mice.

A-C) Merged channels of the *in situ* hybridization of all ligands; *Otx2*, *Wnt1*, and *Fgf8* mRNA probe at low (A) and high (B) magnification, and image captured by confocal microscopy (C).

D) *Otx2* mRNA signal is strong in the mesencephalon and extend as tail to the rostral cerebellar primordium in NTZ.

E) *Wnt1* mRNA signal is highest in the mesencephalon and extend isthmus and as tail to the rostral cerebellar primordium in NTZ.

F) *Fgf8* mRNA signal is present in scattered cells at the rostral cerebellar primordium in NTZ.

G) Panels D, E, F are merged in (G) and blue signal is staining with DAPI.

Abbreviation; cb, cerebellum; m, mesencephalon; NTZ, nuclear transitory zone

Scale bar: 100 µm in A, 20 µm in B, C; 50 µm in D-G

Fig. 6. Fast DiI applied to embryo at E9 and maintained in place for 4 days *in vitro* (DIV 4).

A, a. Fast DiI inserted in mesencephalon at E9 (DIV 0), arrow shows inserted location of DiI crystal in mesencephalon and arrowhead indicate isthmus.

B-C. DiI positive cells directed rostral to mesencephalon (B) and caudally to rostral cerebellar primordium (C).

D-F. low and high magnification shows few cells in the rostral cerebellar primordium in NTZ at level of medial cerebellar section.

G-I. low and high magnification shows few cells in the rostral cerebellar primordium in NTZ at level of lateral cerebellar section.

Abbreviations: cb, cerebellum; m, mesencephalon; NTZ, nuclear transitory zone

Scale bar: 500 µm in a; 200 µm in A; 100 µm in B-D, and G; 50 µm in E, H; 20 µm in F,I

Fig. 7. Fast DiI applied to embryo at E9 and removed after 24 hrs.

A, a. Fast DiI inserted in mesencephalon at E9 (DIV 0) (indicated by arrowhead) and arrow shows the isthmus.

B. DiI positive cells present in cerebellar primordium after DIV 6.

C-D, d. A higher magnification from the caudal to mesencephalon and rostral rhombencephalon shows DiI positive cells in cerebellar primordium

E-G. low and high magnification shows clearly cells with DiI staining in the rostral cerebellar primordium in NTZ after whole mount IHC with NAA and sectioning.

Abbreviations: cb, cerebellum; m, mesencephalon

Scale bar: 500 µm in a, B; 250 µm in A; 200 µm in E; 100 µm in C, D,F; 50 µm in G.

Fig. 8. Sagittal section through cerebellar primordium at E10, double immunofluorescence stained with SNCA and 75ntr

A–C, H. Double immunofluorescence stained with SNCA (**A**, green) and P75 (**B**, red) show co-labeled cells (**C**, merged) in the NTZ and a higher magnification for NTZ is shown in H.

h. Western blotting of SNCA and P75ntr expression during cerebellar development. Immunoblots of total cerebella lysate from embryos at different embryonic days, E11, E13 and E15 indicate an increase in expression of SNCA and P75ntr from E11 to E15. Protein loading was confirmed using βactin.

D–G. Primary dissociated cerebellar culture obtained from E10 mouse embryo, DIV 3, double immunofluorescence stained for SNCA (**D**: green) and P75ntr (**E**: red) and merged (**F**). **G** is a higher magnification of **F**; punctuate immunoreactivity of $SNCA^+$ cells is marked with arrow heads.

I-K. Double immunofluorescence staining of the E10 sagittal section for NAA 3A10 (green) and TrkA (red) shows TrkA immunoreactive cells located in the NTZ.

Abbreviations: cb, cerebellum; NTZ, nuclear transitory zone

Scale bar, 50 µm in A–C and D–F; 20 µm in H and 10 µm in G

Fig. 9. **Sagittal section through cerebellar primordium at E12, double immunofluorescence stained with NAA 3A10 and P75ntr** (we used NAA to determine whether P75ntr fibers are nerve fibers)

A–C, **H.** Double immunofluorescence stained with NAA 3A10 (**A**; green) and P75 (**B**: red) show co-labeled cells and fibers (**C**; merged) in the NTZ and a higher magnification, shown in **H**, indicates the termination of fibers to the subpial surface in rostrocaudal midpoint of the cerebellar primordium.

D–G. Primary dissociated cerebellar culture at E10 mice embryo, after 21 days *in vitro* (DIV 21), double immunofluorescence stained for NAA (green) and P75ntr (red). Immunopositive neuronal somata and

axons shown with NAA 3A10 (**D**), while P75ntr (**E**) immunoreaction is localized in cell membrane that are merged in **F**. **G**. A higher magnification of panel **F**.

Abbreviations: cb= cerebellum, NTZ= nuclear transitory zone

Scale bar, 100 µm in A–C; 50 µm in H and 20 µm in D–F

Fig. 10. Double immunostaining with SNCA and P75ntr at E10 and E12 shows a combination of neurons and fibers present at the core of cerebellar primordium

A–D. Double immunostaining with SNCA (green) and P75ntr (red) at E10 shows more co-labelled cells at the caudal end of the NTZ (**D**, merged; arrow) that continue with fibers that terminate on the subpial surface at the rostrocaudal midpoint of the cerebellar primordium.

E–H. Double immunostaining with SNCA (green) and P75ntr (red) at E12 shows more co-labelled cells at the rostral end of the NTZ (**H**, merged).

Scale bar, 100 µm in A,E; 50 µm in B–D, F–H, I; 20 µm in J–L

Fig. 11. **SNCA peroxidase immunostaining at E14 and P4**

A. Sagittal section of cerebellar primordium at E14 immunostained with SNCA shown weak or lack of SNCA expression in somata.

B, b. are higher magnification for A.

C. A frontal section of the cerebellum at P4 with SNCA immunoperoxidase staining shows that there is no immunoreactivity in the cell body in all three cerebellar nuclei (CN); medial (m), interposed (i) and lateral (l), but it is present distinctly in the axon terminals.

D, E. are higher magnification for A.

Abbreviations: cn, cerebellar nuclei; h, hemisphere; i, interposed nucleus; l, lateral nucleus; m, medial nucleus; 4thv, fourth ventricle; v, vermis

Scale bar, 1000 µm in A; 500 µm in B

Fig. 12. Lack of SNCA expression in the PAP mouse at E12

A–F. Double immunostaining with SNCA (green) and P75ntr (red) at E12 in wildtype (A–C) and PAP-null mice (D–F) shows a lack of SNCA expression in NTZ cells (D), while P75ntr cells are present (E).

Abbreviations: cb, cerebellum; NTZ, nuclear transitory zone

Scale bar, 50 µm in C and F

Fig 13. Double immunofluorescence stained with SNCA (green) and *Lmx1a* (red) shows aggregation of SNCA in embryonic and cerebellar culture

A–D. Primary dissociated cerebellar culture of E10 mice embryo after 5 days *in vitro* (DIV 5), double immunofluorescence-stained with SNCA and βtubulin, shows SNCA localization in cytoplasm and nucleus

E–G. Primary dissociated cerebellar culture of E10, after 6 days *in vitro* (DIV 6), double immunofluorescence-stained for SNCA (green; E) and *Lmx1a* (red; F), and merged in G. The normal appearance of $SNCA^{+}$ cells form a distinct population of CN neurons from the rhombic lip-derived cells ($Lmx1a^{+}$) and are not overlapped.

H–J. Primary dissociated cerebellar culture of E10, after 13 days *in vitro* (DIV 13), double immunofluorescence-stained for SNCA (green; H) and *Lmx1a* (red; I), and merged in (J) shows aggregated form of SNCA in cell or cluster.

Scale bar, 50 µm in E–G; 20 µm in H–J

Fig 14. Double immunostaining for SNCA (green) and cleaved caspase-3 (CC3, red) in the sagittal section of E12 embryo

A-D. Double immunostaining for SNCA (green) and cleaved caspase-3 (CC3, red) in the sagittal section of the E12 embryo shows activation of caspase-3. This indicates dying cells during early cerebellar development. The boxed region in **A** is shown at higher magnification in **B** (SNCA) and **C** (CC3) and merged in **D**, shows activation of caspase-3 in neurons and fiber combinations in the core of the cerebellar primordium

a. Immunoblots of total caspase 3 and cleaved caspase 3 from cerebella lysate of the embryos at different embryonic days, E11, E13 and E15 indicate caspase 3 activation during early cerebellar development

E-G. The lateral aspect of the mouse embryo at E10, whole-mount IHC with NAA 3A10 showing outline of trigeminal ganglion. Double immunostaining for SNCA (green) and cleaved caspase-3 (cc3, red) at sagittal section of E12 embryo trigeminal ganglion shows activation of CC3 is almost localized in the ophthalmic division (op) of the trigeminal ganglion. The boxed region in F is shown at higher magnification in **(G)**. Abbreviations: gg, geniculate ganglion; ntz, nuclear transitory zone; max, Maxillary division of trigeminal ganglion; man, mandibular division of trigeminal ganglion; ot, otocyst; rl, rhombic lip; vg, vestibular ganglion; tg, trigeminal ganglion; 4thV, fourth ventricle; r, rostral; c, caudal; d, dorsal; v, ventral

Scale bar: 50 µm in A,F; 200 µm in E; 20 µm B–D, G

Fig. 1

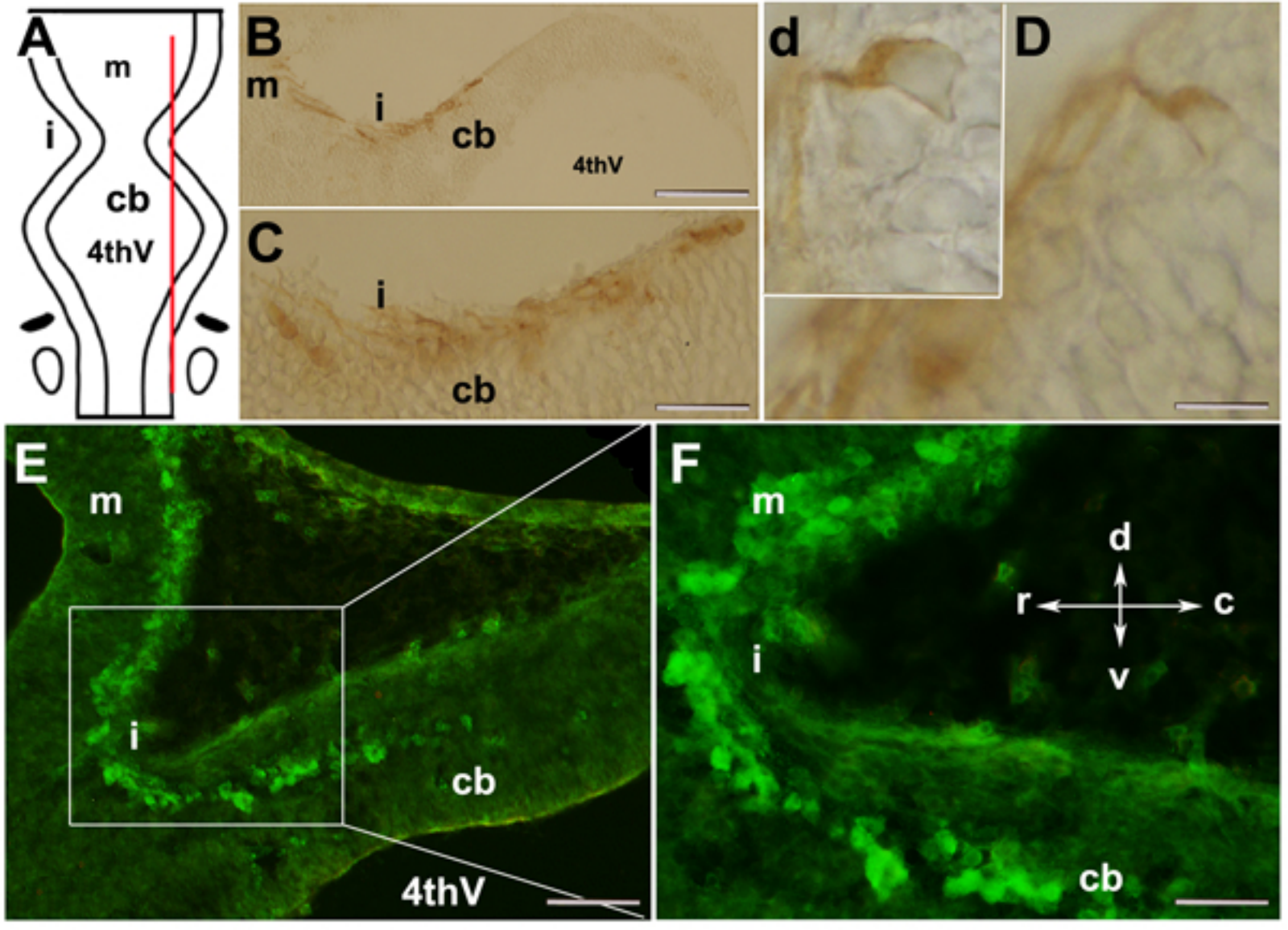
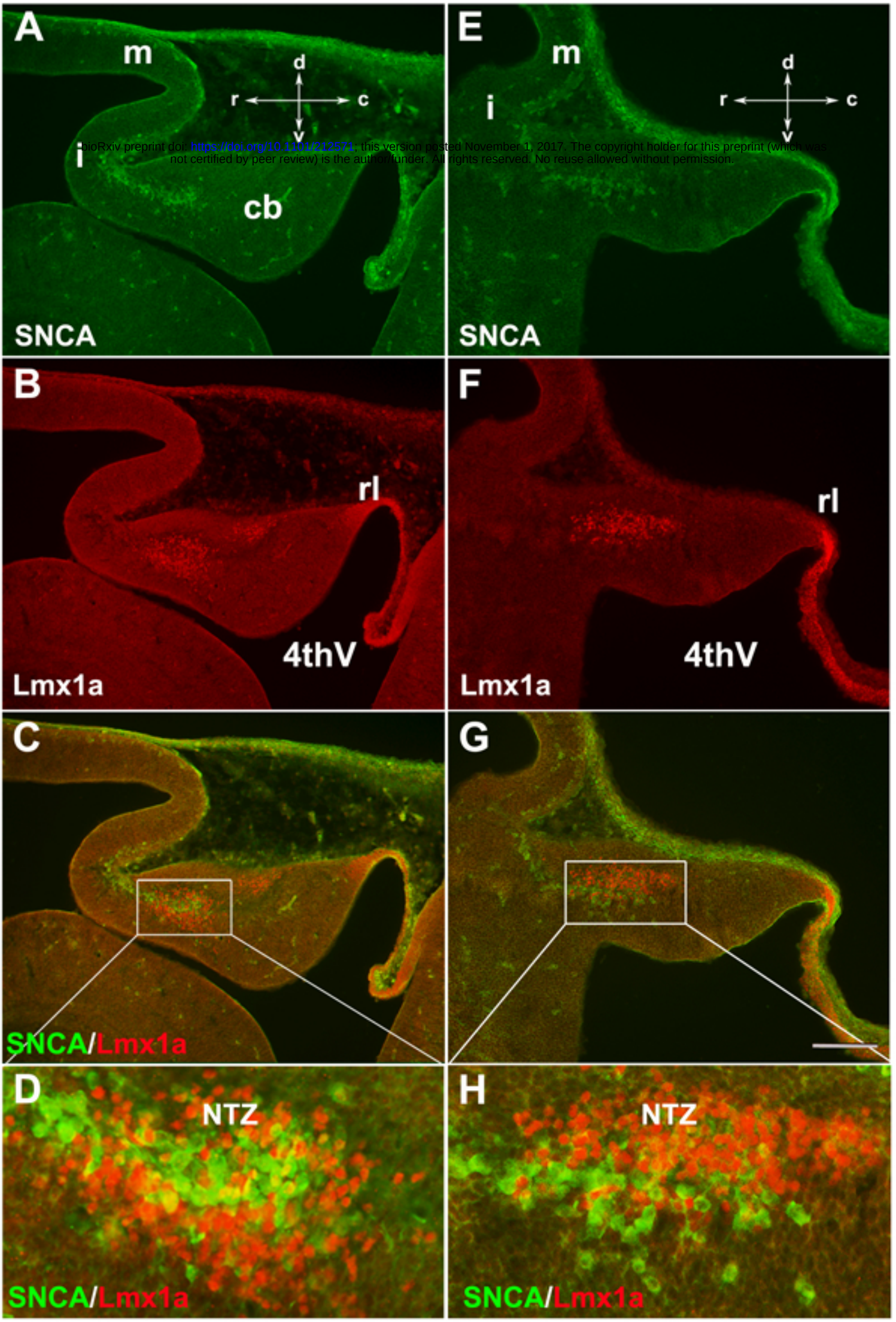


Fig. 2



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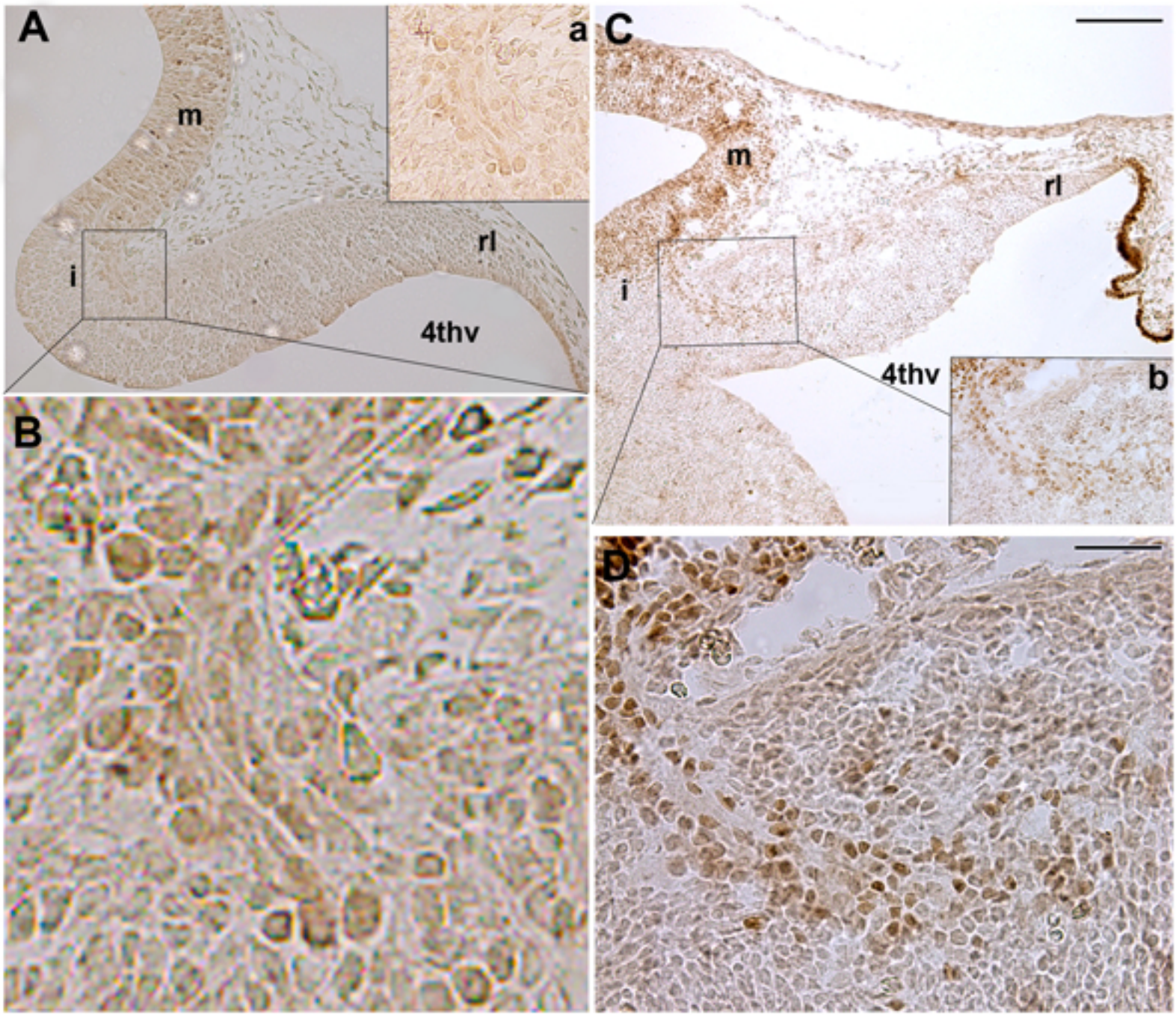

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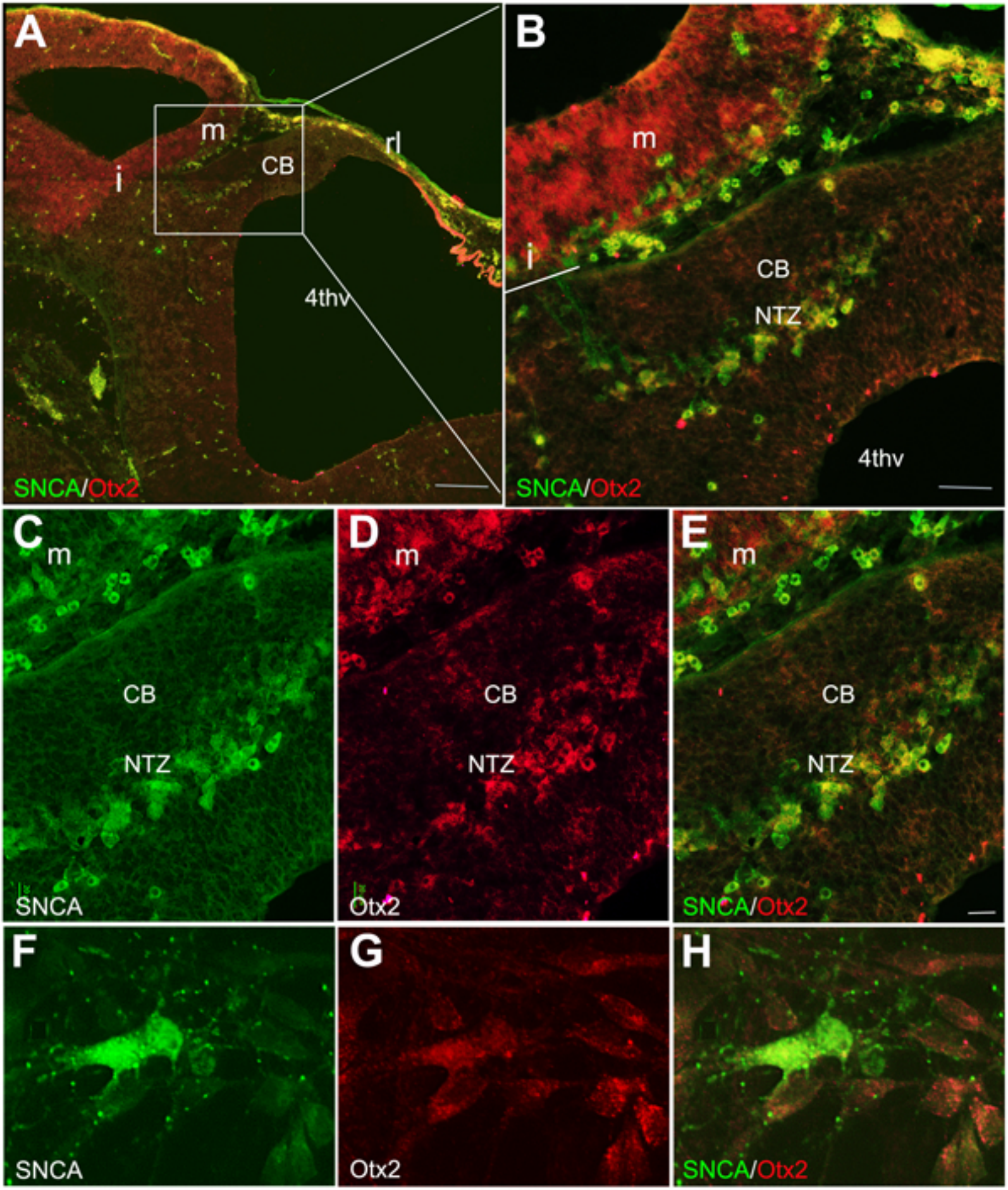

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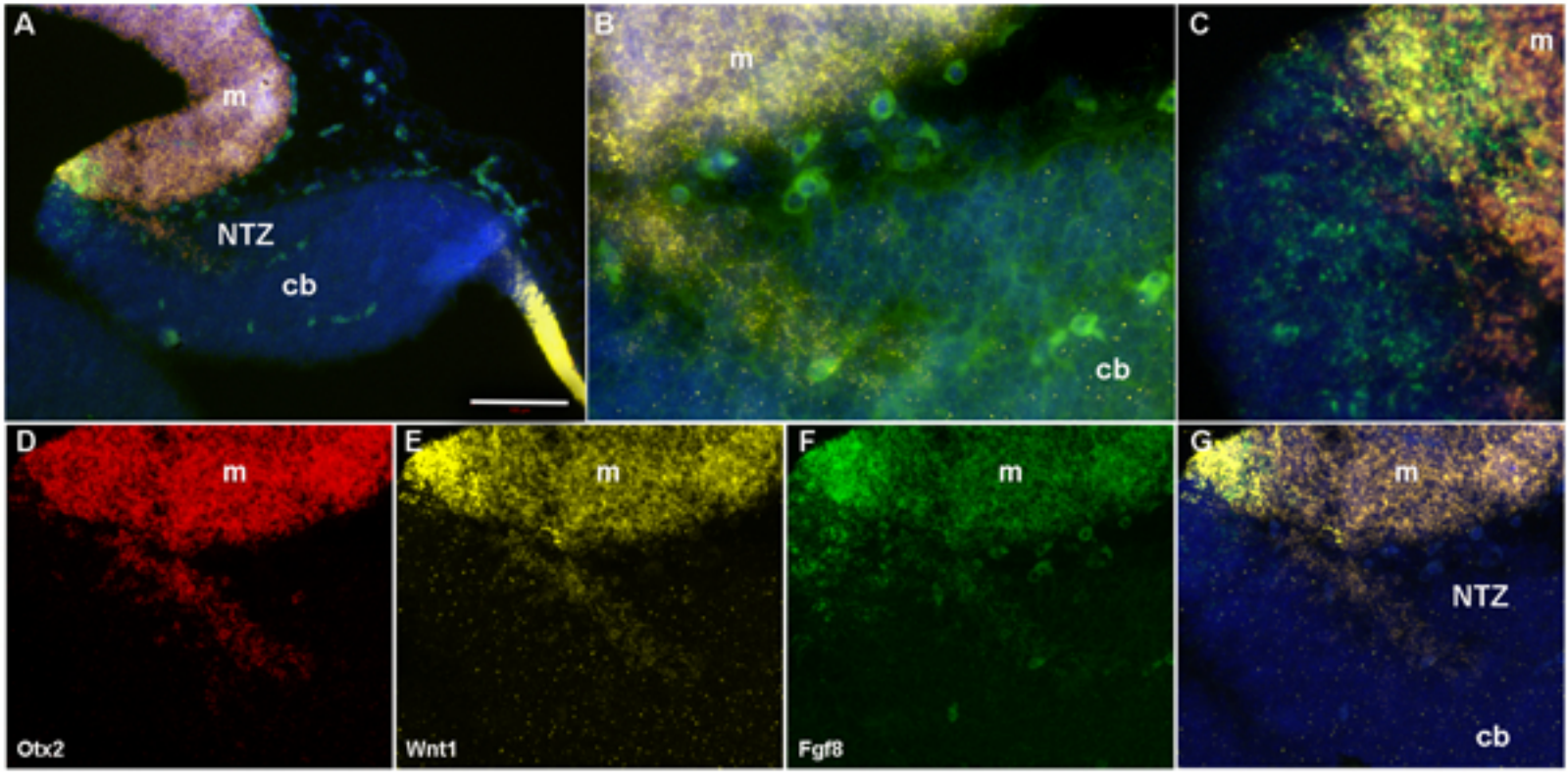

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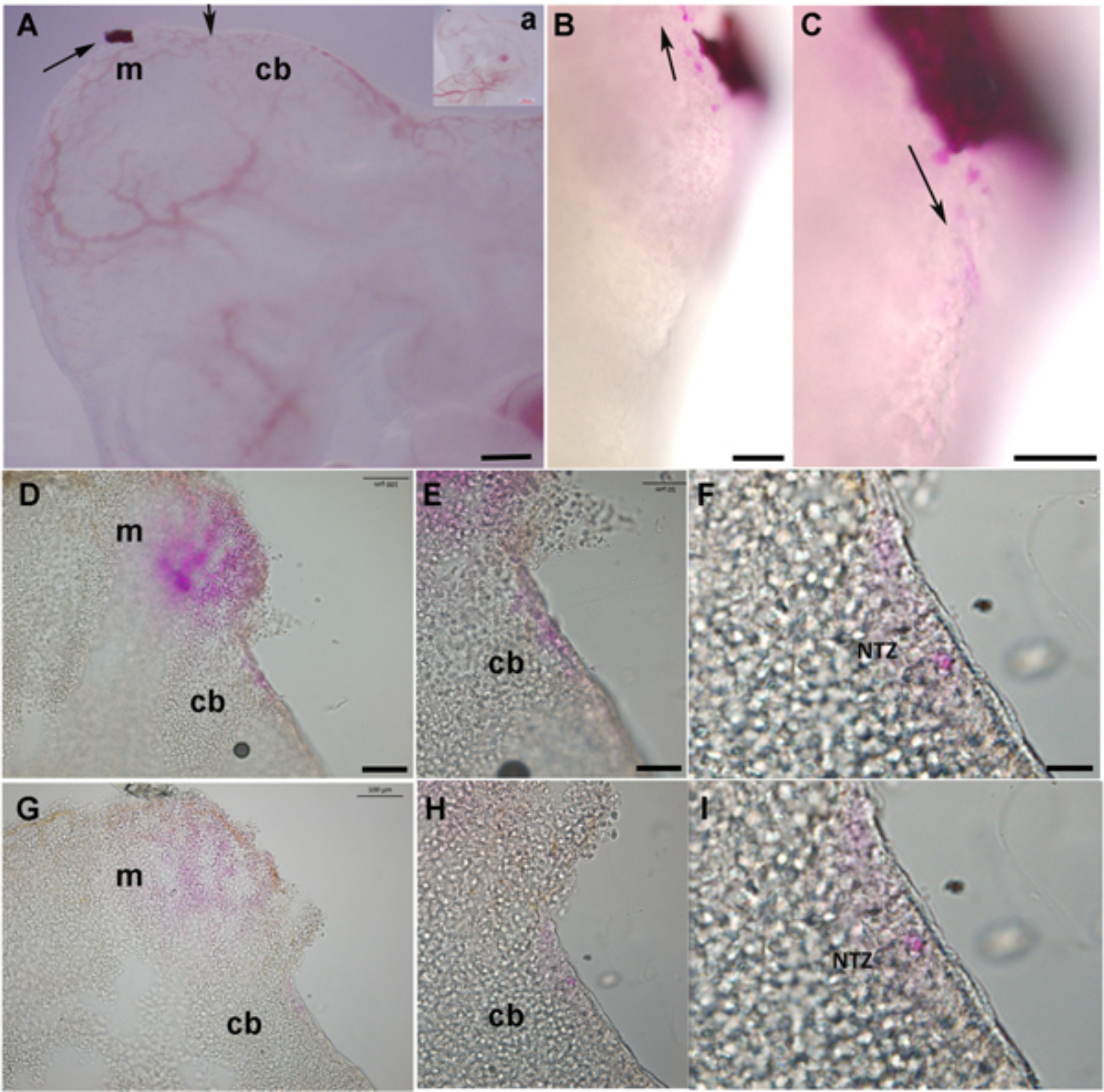

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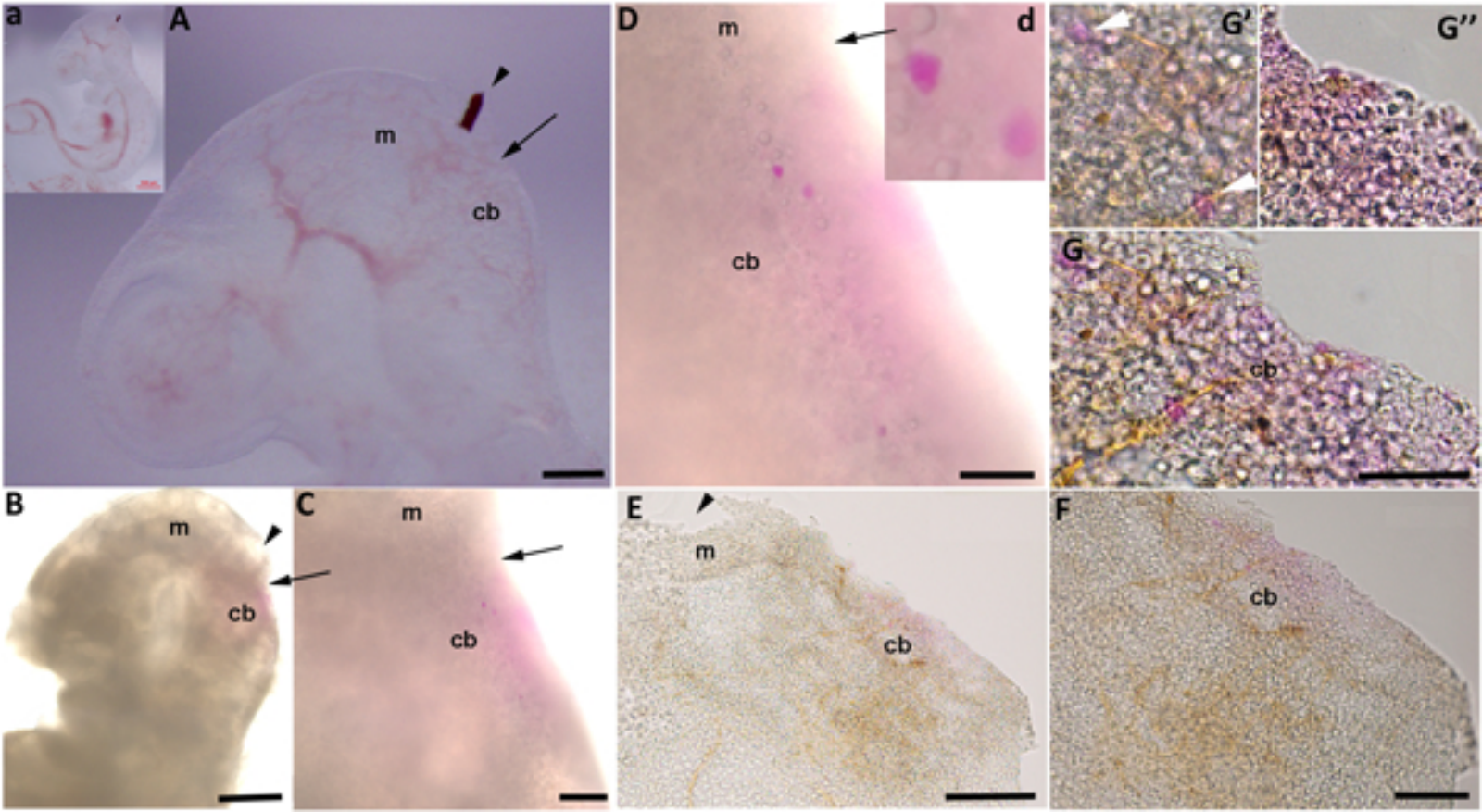

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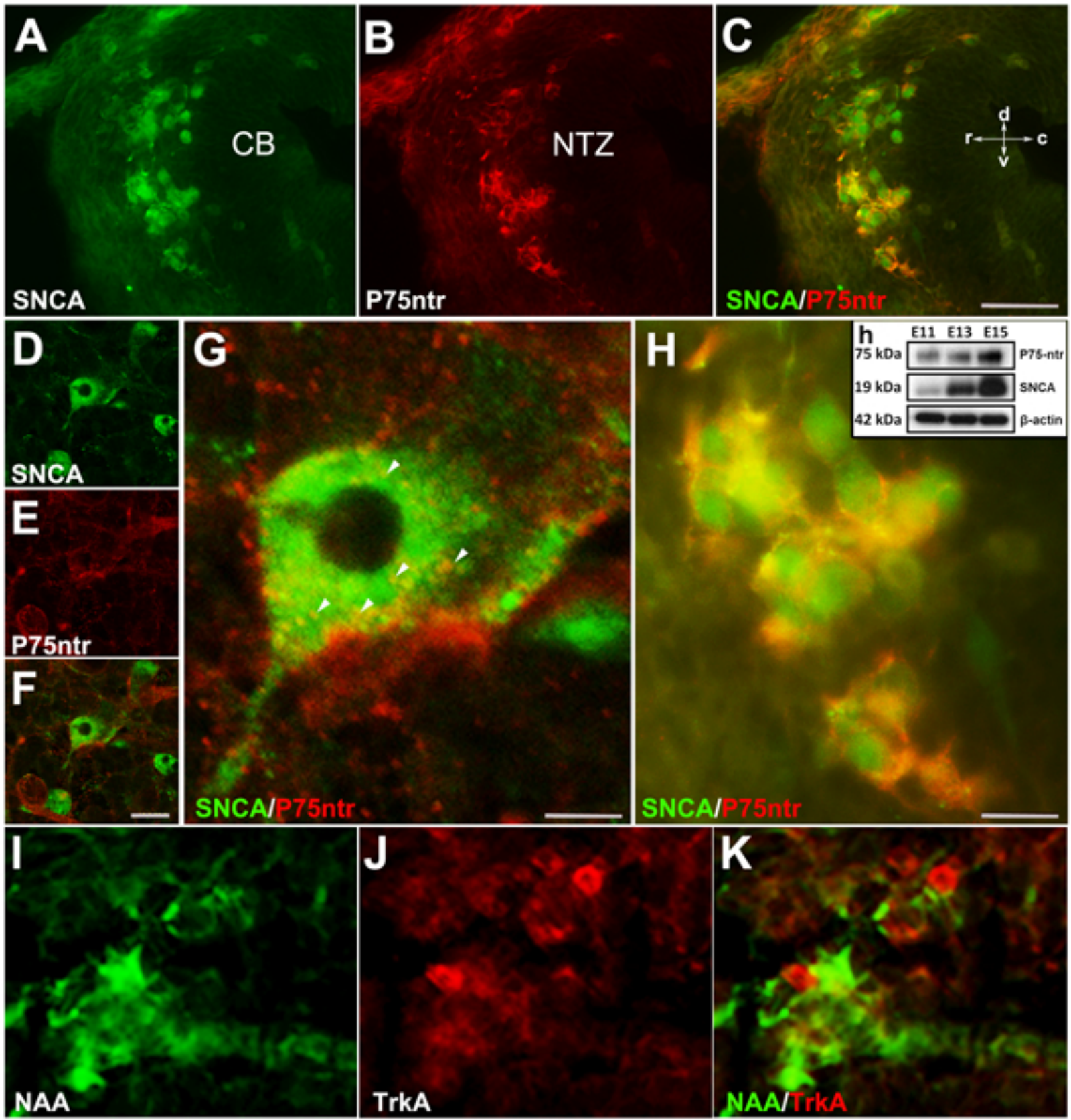
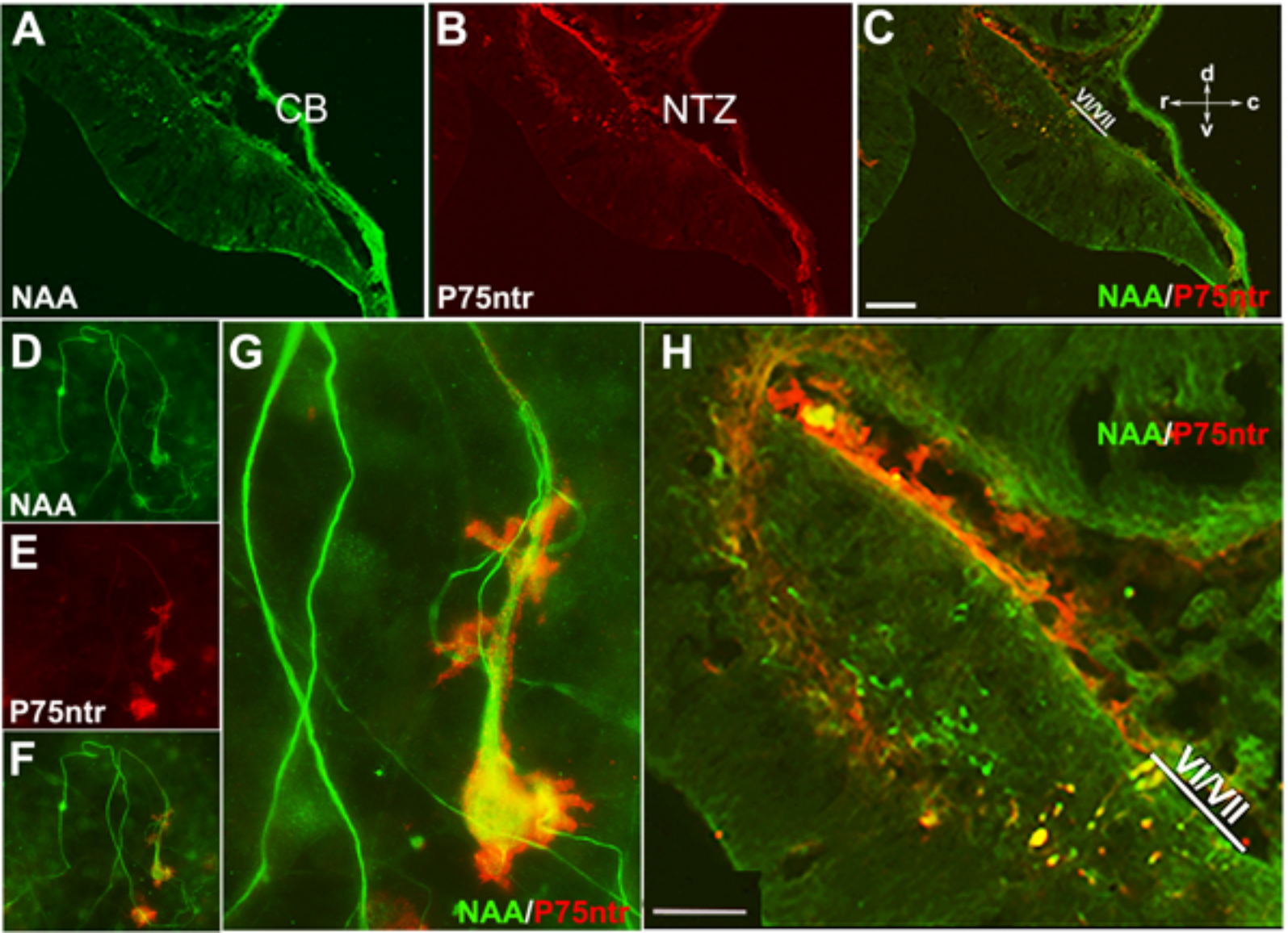

A
CB
SNCA
B
NTZ
P75ntr
C
d
r
c
v
SNCA/P75ntr
D
SNCA
E
P75ntr
F
G
SNCA/P75ntr
H
SNCA/P75ntr
h
E11 E13 E15
75 kDa
P75-ntr
19 kDa
SNCA
42 kDa
β-actin
I
NAA
J
TrkA
K
NAA/TrkA

Fig. 9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B
NTZ
P75ntr
C
d
r
c
v
VIIVII
NAA/P75ntr
D
NAA
E
P75ntr
F
G
NAA/P75ntr
H
NAA/P75ntr
VIIVII

Fig. 10

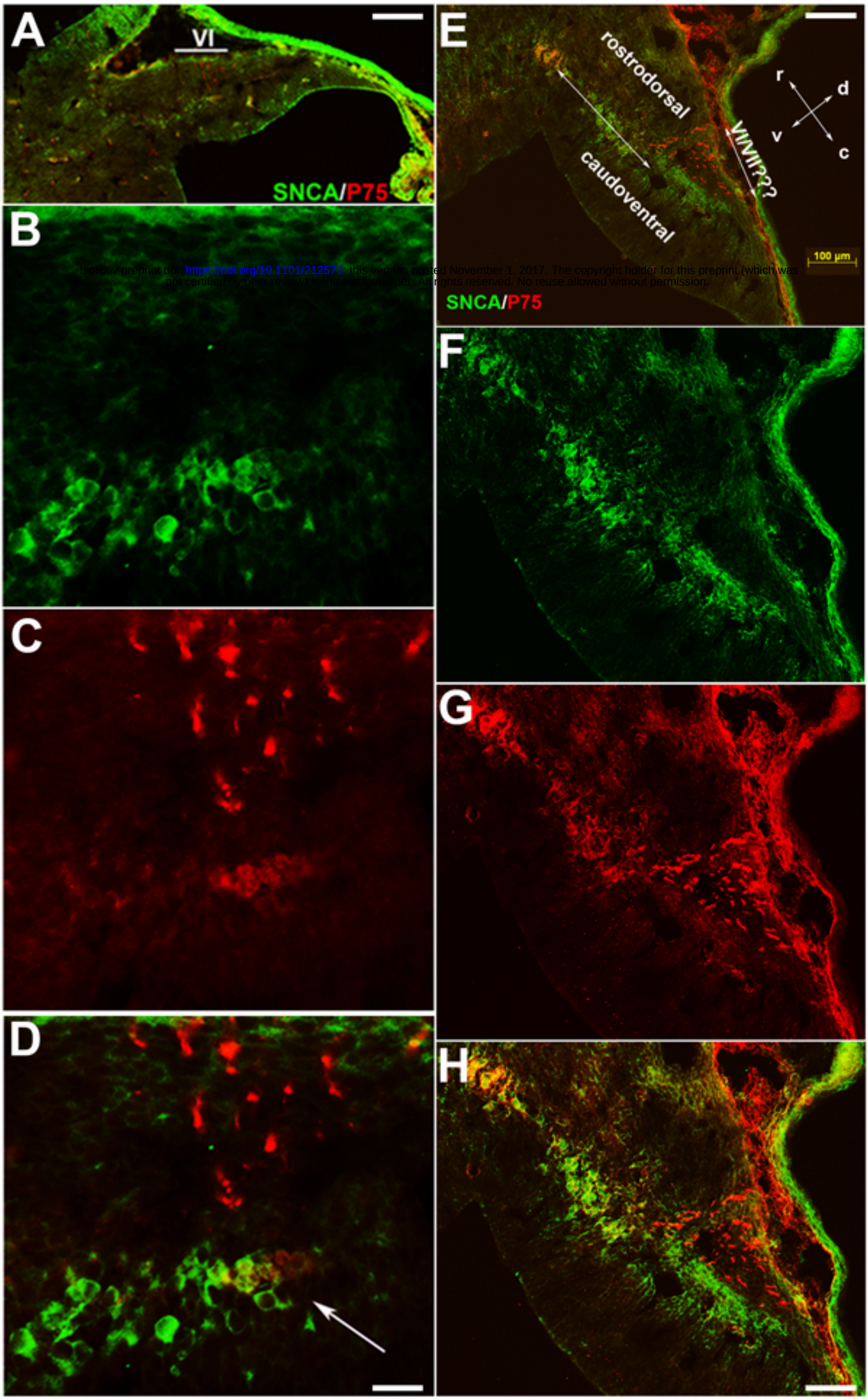


Fig. 11

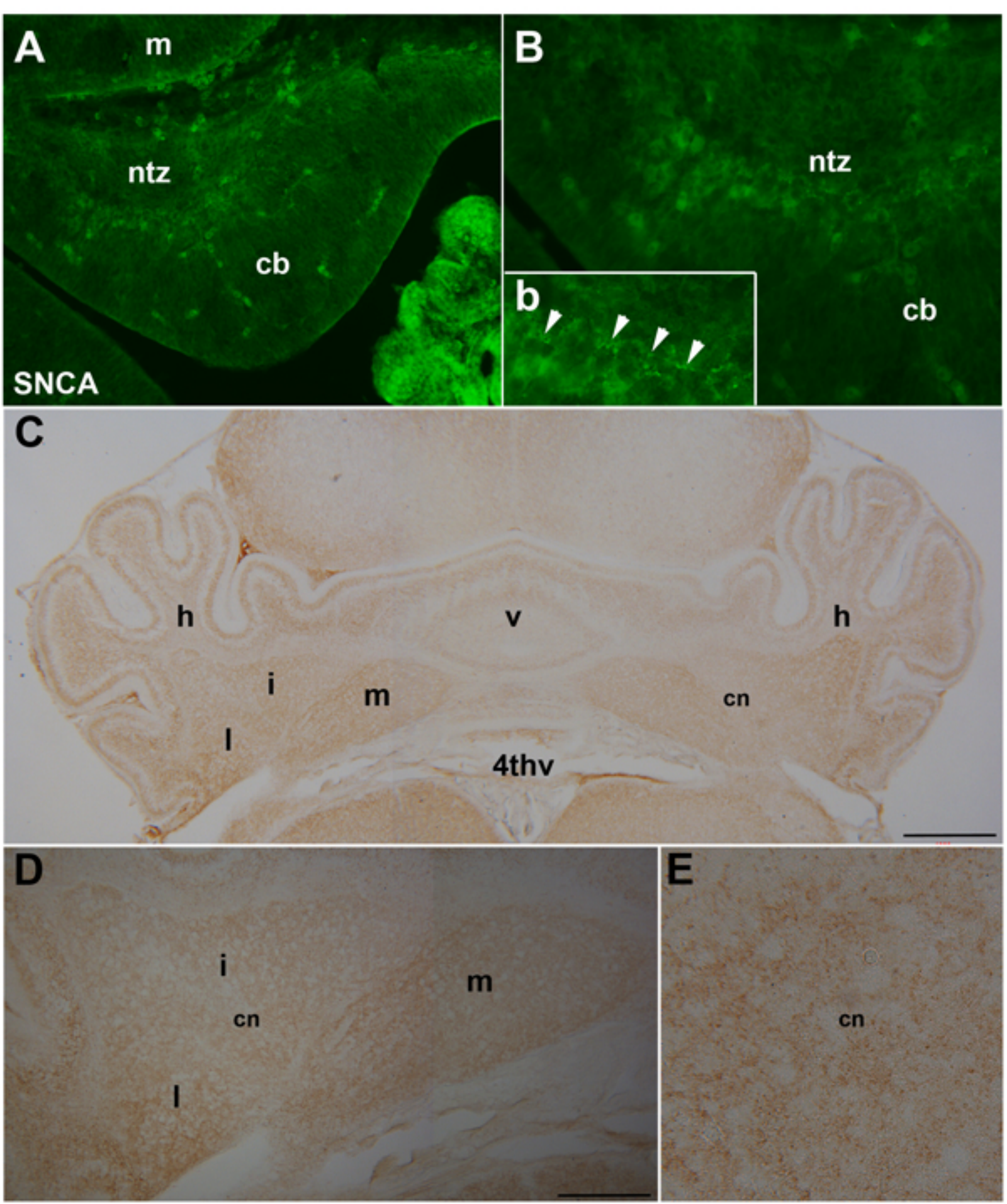
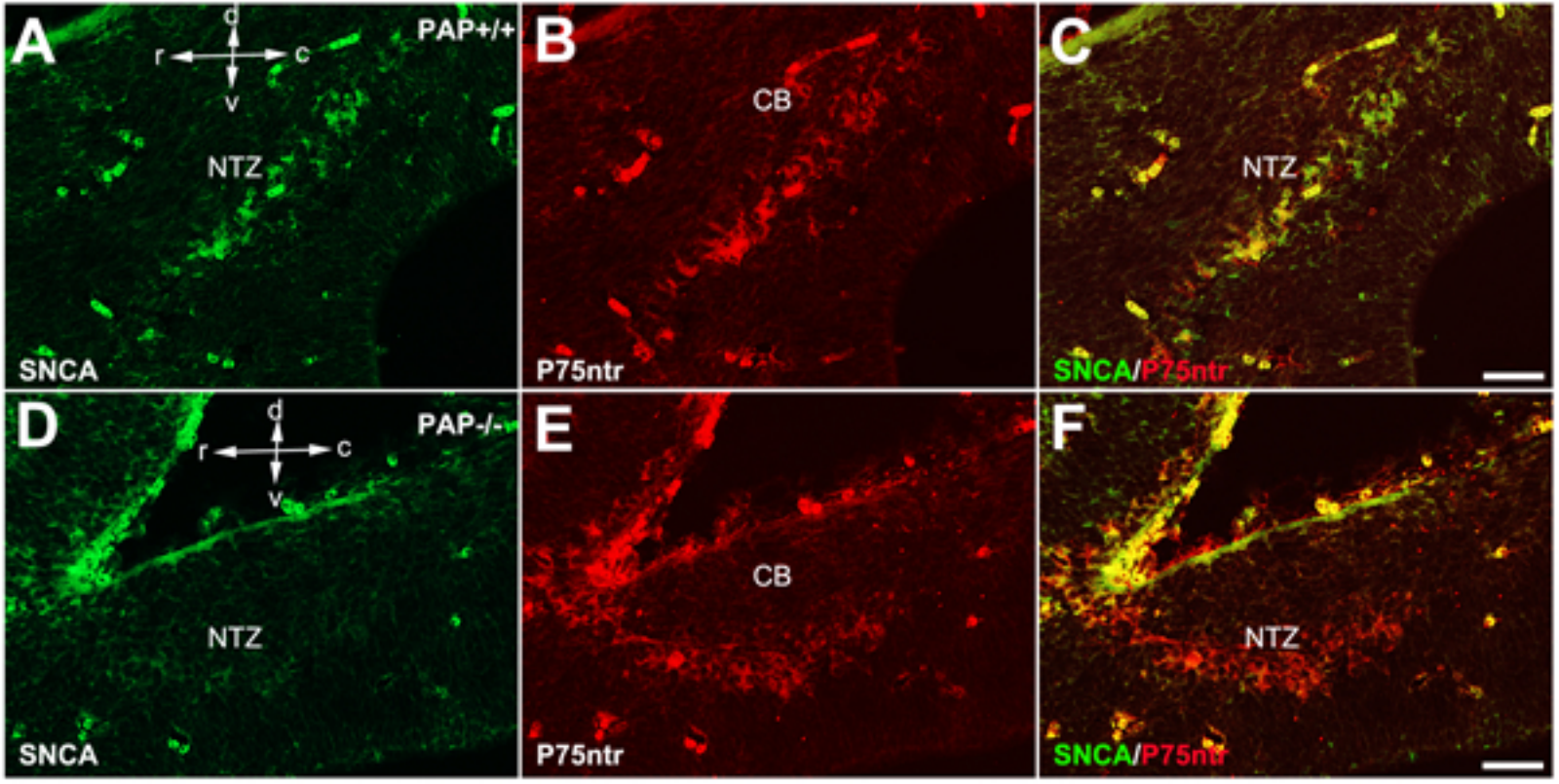


Fig. 12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d
r
c
v
PAP+/+
NTZ
SNCA
B
CB
P75ntr
C
NTZ
SNCA/P75ntr
D
d
r
c
v
PAP-/-
NTZ
SNCA
E
CB
P75ntr
F
NTZ
SNCA/P75ntr

Fig. 13

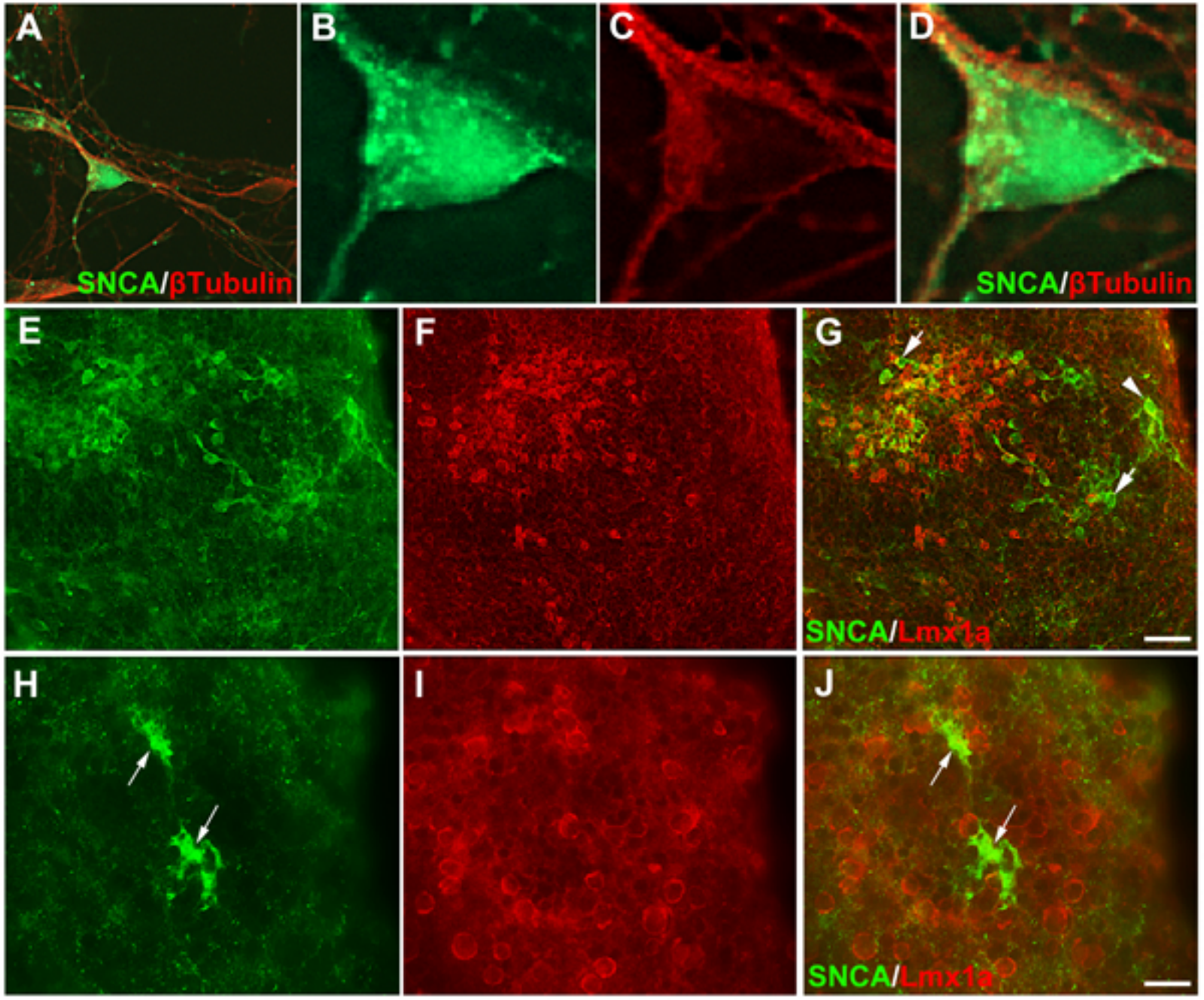
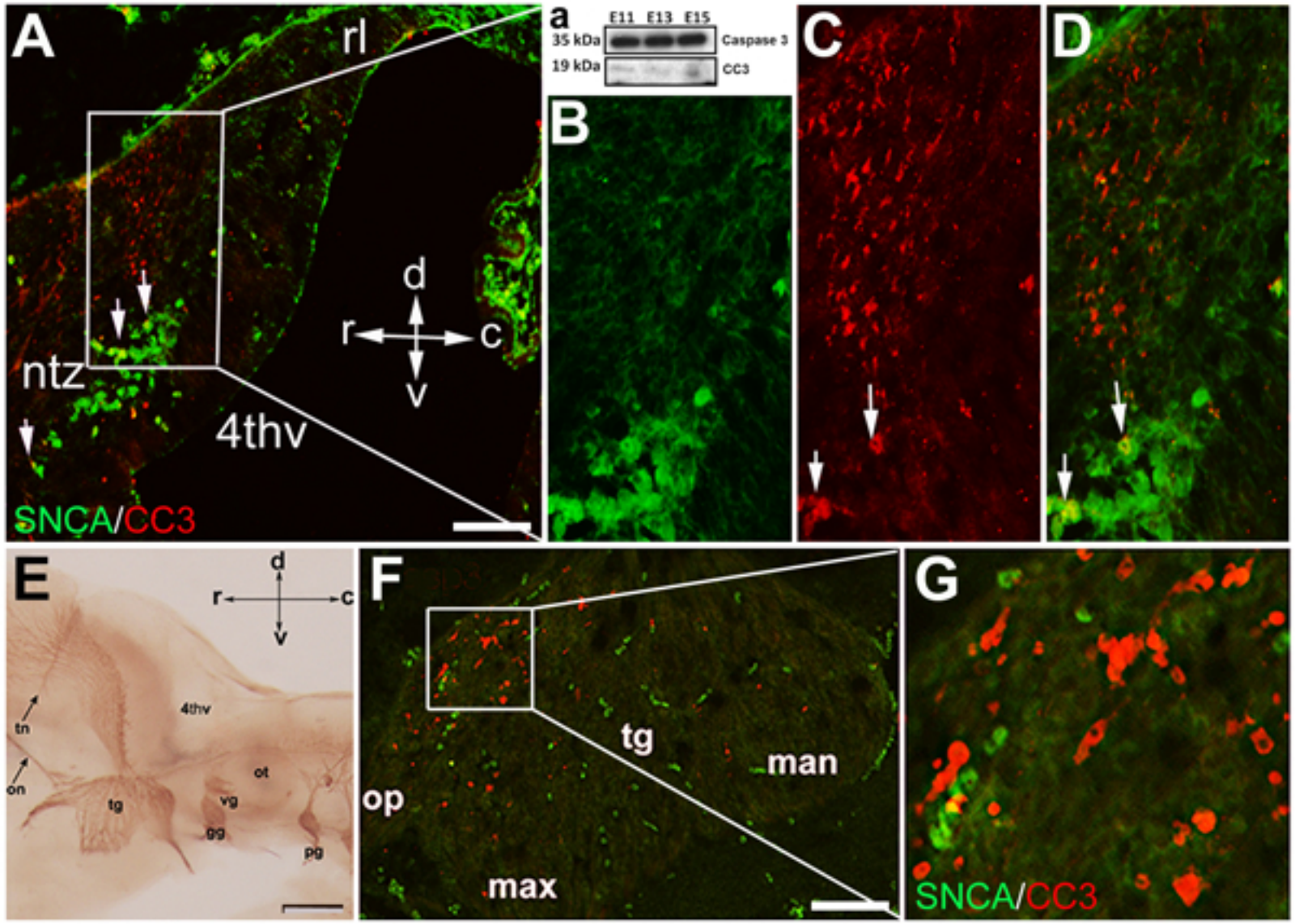

A
B
C
D
SNCA/βTubulin
SNCA/βTubulin
E
F
G
SNCA/Lmx1a
H
I
J
SNCA/Lmx1a

Fig. 14


A
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d
r
c
v
ntz
4thv
SNCA/CC3
a
E11 E13 E15
35 kDa
Caspase 3
19 kDa
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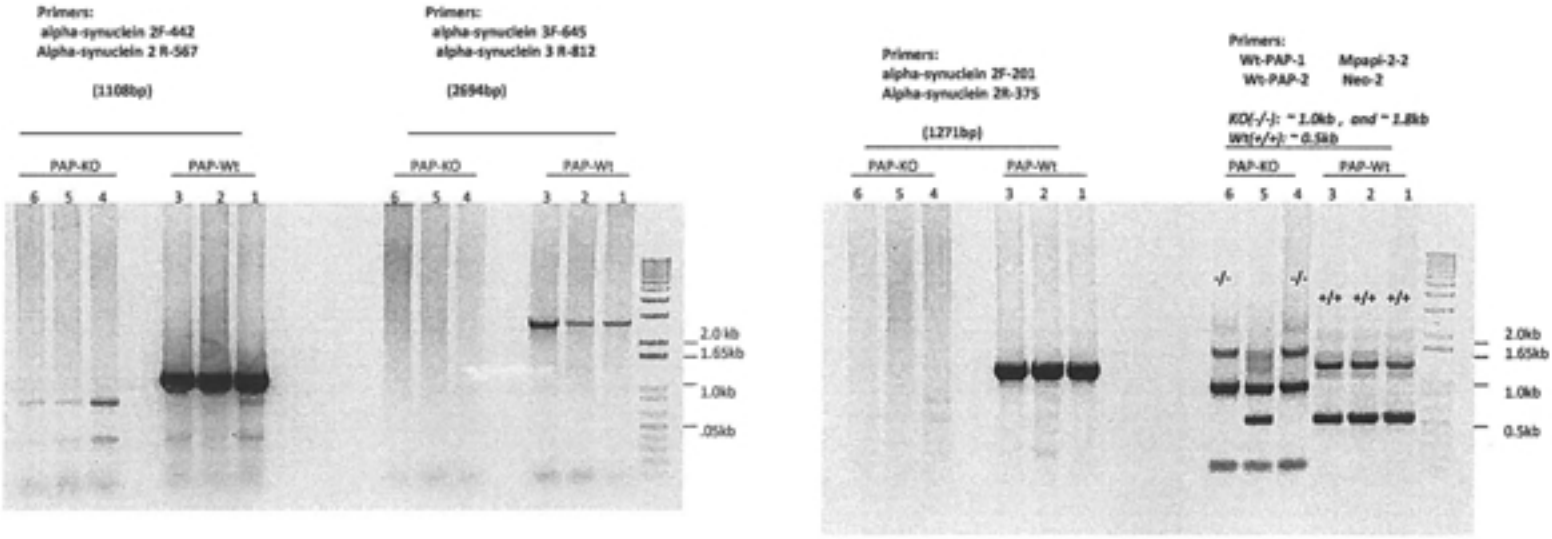
C
D
E
d
r
c
v
4thv
tn
on
ot
tg
vg
gg
pg
F
tg
man
op
max
G
SNCA/CC3

Supplementary 12A



PCR for PAP-Wt and PAP-KO mice genomic DNA (tails)

Primers: ---- Wt-PAP-1 + Wt-PAP-2
In Wt(+/+) :~ 0.5Kb
In PAP KO(-/-) :~ 1.8Kb

----mPAPl2-2 + Neo 3
~ 1.0kb only in PAPKO(-/-)

------alpha-synuclein 2F-201
alpha-synuclein 2R-375 (1271bp)

------alpha-synuclein 2F-642
alpha-synuclein 2R-567 (2694bp)

------alpha-synuclein 3F-645
alpha-synuclein 3R-812 (1108bp)

Dec-03-2013

Samples: mice tail
#1,2,3 : PAP-Wt
#4,5,6 : PAP-KO

Comment:
KO(-/-): ~ 1.0kb , and ~ 1.8kb

Wt(+/+): ~ 0.5kb

Supplementary 12B