The carbohydrate CT1 is expressed in topographically fixed glomeruli in the mouse olfactory bulb

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Abstract

Cell surface carbohydrates define subpopulations of primary olfactory neurons whose axons terminate in select glomeruli in the olfactory bulb. The combination of carbohydrates present on axon subpopulations has been proposed to confer a unique identity that contributes to the establishment of the olfactory topographic map. We have identified a novel subpopulation of primary olfactory neurons in mice that express blood group carbohydrates with GalNAc-ß1,4[NeuAcα 2,3]Galß1 residues recognised by the CT1 antibody. The CT1 carbohydrate has been shown to modulate adhesion of nerve terminals to the extracellular matrix and to synaptic proteins. The axons of the CT1-positive primary olfactory neurons terminate in a subpopulation of glomeruli in the olfactory bulb. Four lines of evidence support the view that CT1 glomeruli are topographically fixed. First, CT1 glomeruli were restricted predominantly to the dorsomedial olfactory bulb and were absent from large patches of the ventrolateral bulb. Second, similar distributions were observed for CT1 glomeruli on both the left and right olfactory bulbs of each animal, and between animals. Third, CT1 glomeruli were typically present as small clusters of 2-4 glomeruli. Fourth, a single CT1 glomerulus was always apposed to the glomeruli innervated by axons expressing the M72 odorant receptor. We also show that the CT1 carbohydrate is lost in gain-of-function transgenic mice over-expressing the blood group A glycosyltransferase in which there is aberrant targeting of M72 axons. Taken together, these results suggest that the CT1 carbohydrate, together with other carbohydrates, contributes to axon guidance during the establishment of the olfactory topographic map.

1. Introduction

In the mammalian olfactory system, primary olfactory neurons each express one of ~1000 odorant receptors and are distributed mosaically throughout the olfactory epithelium (Buck and Axel, 1991). The axons that extend from the primary olfactory neurons fasciculate together in bundles of mixed axons, and project to the olfactory bulb. At this point they defasciculate and sort so that axons expressing the same odorant receptor target specific glomeruli located in roughly topographically fixed positions on the olfactory bulb surface. We have previously proposed a hierarchical model of axon guidance to explain how axons from widely dispersed neurons expressing the same odorant receptor are able to converge and form glomeruli (Key and St John, 2002). Most of the predictions of this model including the presence of zonal cues (Bozza et al., 2009), the complementary gradients of axon guidance ligands and their receptors (Cho et al., 2007; Takeuchi et al., 2010) and mosaically expressed sorting cues (Cutforth et al., 2003; Kaneko-Goto et al., 2008; Serizawa et al., 2006) have been confirmed. While we and others have revealed that the mosaic expression of cell surface carbohydrates plays a role in axon navigation in the olfactory system (Lipscomb et al., 2002; Lipscomb et al., 2003; Schwarting and Henion, 2007; St John et al., 2006), most likely through selective fasciculation of axons involving lectin-like molecules (Puche and Key, 1996; St John and Key, 1999; Storan et al., 2004), there is very little evidence suggesting that the expression of these molecules is sufficiently fine-tuned to account for the observed topography in the primary olfactory pathway.

Carbohydrates are expressed by primary olfactory axons and their differential distribution provides a potential mechanism for conferring a unique identity to each

subset of primary olfactory neurons, or a "glycocode" (St John and Key, 2005). Some carbohydrates are universally expressed by primary olfactory neurons whereas others are expressed by large subpopulations of neurons. For example, blood group H carbohydrate is expressed on all axons (St John et al., 2006) while the NOC-7 carbohydrate with an α-extended lactoseries (NOC-7), recognised by the LA4 antibody, is widely expressed (Storan et al., 2004). In contrast, the N-acetyl-D-lactosamine carbohydrate recognised by *Dolichos biflorus* agglutinin (DBA) (Key and Akeson, 1993; Lipscomb et al., 2003), the lactosamine glycan LCG (Crandall et al., 2000), the NOC-8 carbohydrate recognised by KH10 (Storan et al., 2004), the NOC-3 carbohydrate (St John and Key, 2001) and the *N*-acetyl-galactosamine linked alpha or beta to the 3 or 6 position of galactose recognised by the lectin *Wisteria floribunda* agglutinin (WFA) (St John and Key, 2002) are expressed by large subsets of neurons. We postulate that the differential expression of carbohydrates by smaller subpopulations of primary olfactory neurons may provide a mechanism for axons to be uniquely coded and to assist in sorting en route to their target glomeruli. One of the predictions of this hypothesis is that axons expressing these carbohydrates will terminate in topographically fixed locations in the olfactory bulb.

To test this idea we examined the expression of Cad/Sda blood group carbohydrate antigens (GalNAc-ß1,4[NeuAcα 2,3]Galß1 residues), which are recognised by CT1 (cytotoxic T cells) antibodies (Lefrancois and Bevan, 1985), in the mouse primary olfactory system. In neuromuscular junctions, the CT1 carbohydrate is expressed in nerve terminals on glycoproteins that modulate adhesion to the extracellular matrix (Xia et al., 2002; Xia and Martin, 2002) and, as such, is a strong candidate for modulating axon guidance in the olfactory system. We found here that the CT1

carbohydrate is expressed by a novel subpopulation of primary sensory olfactory neurons including their axons and their glomerular terminations in the olfactory bulb. The glomeruli innervated by the CT1 expressing axons were present predominantly in the mediodorsal surface of the olfactory bulb in what appeared to be topographically fixed positions. These results support the view that differential expression of carbohydrates generates a glycocode that confers a unique identity for primary olfactory axons.

2. Materials and methods

2.1 Animal preparation

Wild type Quackenbush mice, M72-IRES-tau-GFP (Potter et al., 2001), P2-lacZ mice (Mombaerts, 1996), BGAT-Tg mice (St John et al., 2006) were examined. Three to six animals were examined for each age and for each mouse line. Embryonic and postnatal mice were decapitated, adult mice were asphyxiated by CO_2 and heads were immersion fixed overnight at 4ºC in 4% paraformaldehyde. Following fixation, postnatal heads were cryoprotected in 30% sucrose and adult heads were decalcified in 20% disodium ethylene diaminetetraacetic acid in PBS (pH 7.4). Heads were placed in embedding matrix (O.C.T. compound, Miles Scientific, Naperville, IL), snap frozen by immersion in iso-pentane that had been cooled by liquid nitrogen and sectioned (30 μm) on a cryostat microtome.

2.2 Histochemistry

Immunohistochemistry and lectin histochemistry was performed as previously described (St John et al., 2006). Monoclonal antibodies against CT1 (Conzelmann and Lefrancois, 1988; Lefrancois and Bevan, 1985) were prepared in the laboratory of Prof Paul Martin from hybridoma cells provided by Leo Lefrancois. The CT1 antibodies have been previously demonstrated to react with GalNAc and sialic acid containing antigens defined as GalNAcß1,4-[SAα2,3]Gal carbohydrate determinants. This was demonstrated by showing specific binding to the murine CTL-clone B6.1, the human blood group Cad antigen, and the Tamm-Horsfall urinary glycoprotein from Sda[+] individuals; when the three different sources lacked the GalNAc or sialic acid residues there was no immunoreactivity by the CT1 antibodies (Conzelmann and Lefrancois, 1988). Sections were incubated with anti-CT1 followed by horse anti-

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mouse Alexa Fluor[488] (1:400; Molecular Probes, Carlsbad, CA) or anti-mouse

μ–chain specific conjugated to Texas Red (1:200, Abcam ab5927); or were incubated

with anti-GFP or anti-beta galactosidase followed by goat anti-rabbit secondary

antibodies conjugated to biotin (1:200; Vector Labs, Burlingame, CA) and then with

Streptavidin-conjugated Alexa Fluor[488] (1:400; Molecular Probes). Alternatively,

immunostaining staining was visualised using avidin-biotin-horseradish peroxidase

(Vector Laboratories Inc) followed by reaction with 3,3'-diaminobenzidine (DAB)

and hydrogen peroxide (H_2O_2) in TBS. For lectin staining, sections were incubated

with biotinylated *Dolichos biflorus* agglutinin in BSA/TBS/Triton X-100 with 1.25

mM $CaCl_2$, 1.25 mM $MgCl_2$ and 1.25 mM $MnCl_2$. The sections were then washed in

TBS/Triton X-100 and incubated with either (i) avidin-biotin-horseradish peroxidise

for 1 h at RT with staining visualised by reaction with diaminobenzidine as described

above, or (ii) Streptavidin-conjugated Alexa Fluor[488] as described above.

2.3 Imaging

Images of the sections were captured using Olympus BX51 compound fluorescence

microscope fitted with differential inference contrast. Images were colour balanced in

Adobe Photoshop CS5 with uniform colour and gamma adjustment and figures were

complied using Adobe Illustrator CS5 (Adobe Systems Incorporated).

2.4 Quantification of CT1 neurons

The percentage of mature neurons that expressed CT1 in adult animals was

determined using coronal sections from OMP-ZsGreen transgenic mice that were

immunostained using anti-CT1 antibodies. Five sections from each animal (n=3

animals) were selected with each section separated by 300 μm so that the sections

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covered the rostral-caudal range of the olfactory epithelium. For each section, all regions of the epithelium were included in the quantification. The number of ZsGreen neurons that co-expressed CT1 and the total number of ZsGreen neurons were counted to determine the proportion of mature neurons that expressed CT1.

2.5 Mapping of glomeruli

CT1 immunostaining was performed on sections from postnatal day 7 animals to prepare three maps from different animals. Every 4th coronal section from the rostral tip of the olfactory bulb to the caudal region of the accessory olfactory bulb was used. Sections were stained using diaminobenzidine with the reaction time being the same for each section. For each section a vertical line was drawn from the most ventral portion of the bulb to the mid point of the dorsal surface, with the dorsal point serving as the position from which the butterfly schematic preparation was opened. The circumference of the outer surface of the olfactory bulb was measured for each section to generate the shape of the butterfly schematic of the olfactory bulb. The positions of each CT1-positive glomerulus were measured from the dorsal point and then mapped onto the schematic butterfly preparation of the olfactory bulb. Glomeruli were categorised as having strong expression or weak expression or no expression of CT1 using a binarised threshold level in ImageJ. Glomeruli with strong or weak expression were mapped onto the schematic. A consensus map was not appropriate due to the low number of olfactory bulbs that were mapped (n=3), the sampling of every 4th section and the local variability in the position of individual glomeruli. Maps appeared similar and the map from a single animal is presented in Figure 5. The strong and weak expressing CT1 glomeruli were counted to determine the proportion of glomeruli within each region.

3. Results

3.1 CT1 carbohydrate is expressed by a unique subpopulation of primary olfactory neurons

The expression of CT1 carbohydrate was examined using immunohistochemistry on cryostat sections of the primary olfactory system from both wild-type mice and OMP-ZsGreen transgenic mice which express ZsGreen, a variant of green fluorescent protein, under the control of the OMP promoter (Ekberg et al., 2011; Windus et al., 2010). The robust fluorescence of primary olfactory neurons in the OMP-ZsGreen mice provides a simple means for characterising the lineal expression of the CT1 epitope in the olfactory system. OMP is a marker of more mature differentiated neurons in the olfactory epithelium (Baker et al., 1989). CT1 expression was first detected on the dendrites, perikarya and axons of a subset of OMP negative primary olfactory neurons at E18.5 (Fig. 1A-B). By postnatal day 7 (P7), the expression of CT1 had increased and there were now many OMP positive neurons co-expressing this carbohydrate antigen (Fig. 1C-E). A small subset of these CT1-positive neurons was still not expressing OMP (Fig. 1E, arrows). In adults, the CT1 positive neurons were stochastically dispersed throughout all regions of the olfactory epithelium in the nasal cavity (Fig. 1F-H), but the density of stained cells was clearly not uniform with some regions having higher density of CT1 neurons (arrows in Fig. 1G) while other regions had lower density (arrow with tail, Fig. 1G). We estimated the proportion of neurons that expressed CT1 by counting cells throughout the nasal cavity in coronal sections that covered the medial-lateral and rostral-caudal range of the nasal cavity. We found that around 15-20% of olfactory neurons (identified by expression of ZsGreen) expressed the CT1 epitope in the adult.

Within the olfactory bulb, the CT1 positive axons projected to clusters of glomeruli positioned predominantly at the medial and dorsal margins along the rostral-caudal length of the bulb (Fig. 2A-C). In these regions, the CT1 glomeruli typically appeared discretely clustered as pairs or quadruples which were often separated by unstained glomeruli. These clusters contained glomeruli which were strongly (arrows, Fig. 2D-H) or weakly (arrowheads, Fig. 2D-H) stained. Only in regions containing few stained glomeruli were isolated single-stained glomeruli evident (unfilled arrowhead, Fig. 2C; arrow with tail in Fig 3A, B).

The restricted expression pattern of CT1 in glomeruli suggested that the CT1 glomeruli were in topographically similar positions. We confirmed this by examining coronal sections of both the left and right olfactory bulbs which were in the same rostral-caudal plane. However, it should be noted that the positions of CT1 glomeruli between the left and right olfactory bulbs, while appearing similar, were not exact mirror images. This is due to both the difficulty in obtaining perfectly coronal planes as well as to known local variability in the positions of glomeruli expressing the same odorant receptor in different bulbs (Schaefer et al., 2001). Nevertheless, distinct glomeruli were observed in topographically similar positions between the left and right olfactory bulbs in all five postnatal day 7 animals that were examined. In the rostral olfactory bulb, CT1 glomeruli were predominantly on the medial surface of the bulb and numerous glomeruli appeared to be in similar topographical positions in both bulbs (arrows, Fig. 3A-B). In the central region of the olfactory bulb, CT1 glomeruli tended to be present in the dorsal region; and glomeruli had similar topographical locations in both bulbs (Fig. 3D-E). In the caudal region of the olfactory bulb, CT1

glomeruli were scattered throughout all surfaces, again with glomeruli having similar topographical positions in both bulbs (Fig. 3G-H).

The distribution of the CT1 glomeruli was reminiscent of the distribution of the glomeruli stained by the lectin *Dolichos biflorus* agglutinin (DBA) (Key and Akeson, 1993). We next compared the distribution of CT1 glomeruli to that of DBA glomeruli (Fig. 3). The DBA glomeruli were scattered around the olfactory bulb and were intermingled with glomeruli that did not express DBA ligands (Fig. 3C, F and I). Although the CT1 glomeruli were located in positions similar to those of the DBA glomeruli (arrows, Fig. 3C, F and I), it was clear that there were many more DBA stained glomeruli in the olfactory bulb. We next used double staining to show that the CT1 glomeruli were a subset of the DBA reactive glomeruli (Fig. 3J-L). Thus the CT1 and DBA bound carbohydrates are clearly molecularly distinct and CT1 is expressed by a subset of the neurons that expresses the carbohydrate recognised by DBA.

3.2 CT1 is expressed in the accessory olfactory system during development
We next examined CT1 expression in the accessory olfactory system. In postnatal day 7 animals, a small number of CT1-positive neurons were present in the basal layer of the vomeronasal epithelium (Fig. 4A-D). Within the accessory olfactory bulb, the CT1-positive axons projected to the caudal region where they terminated in a few glomeruli some of which exhibited strong expression of CT1 while others expressed lower levels (Fig. 4E). This topography is consistent with previous reports indicating that basal neurons which express the V2R family of vomeronasal receptors project to the caudal accessory bulb (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Thus it appears that the CT1 expressing neurons represent

a unique subpopulation of the V2R receptor-expressing neurons. In the adult, CT1 was neither expressed by neurons in the vomeronasal organ (data not shown) nor by axons and terminals in the accessory olfactory bulb (Fig. 4F). In contrast, the lectin DBA stained all vomeronasal axons within the accessory olfactory bulb (Fig. 4G).

3.3 Topography of CT1 glomeruli

To obtain a better understanding of the distribution of the CT1-positive glomeruli in the main olfactory bulb, we mapped the positions of the glomeruli in postnatal day 7 animals by projecting them onto a butterfly schematic preparation of the olfactory bulb. As observed in the coronal sections (Fig 2D-H), glomeruli with strong CT1 expression were adjacent to glomeruli with weak CT1 expression and to glomeruli that did not express CT1. We used two qualitative categories of CT1 expression: high and low which were based on the intensity of staining visualised by the diaminobenzidine reaction; glomeruli that did not express detectable levels of CT1 were not mapped. Two-thirds (64% +/- 0.2%, n=3) of the CT1 glomeruli were present on the medial surface of the olfactory bulb with the majority (70%) being in the dorsal half of the bulb (Fig. 5). Within the ventral olfactory bulb, the CT1 glomeruli were sparsely distributed and were often isolated from other CT1 glomeruli. The majority (75%) of the CT1 glomeruli in the ventral region were categorised as having low CT1 staining. In the dorsal olfactory bulb, there was an equal proportion of high and low expressing CT1 glomeruli. Schematic maps from three different animals were generated and showed overall similarities, yet exhibited minor positional differences for individual glomeruli as expected (Schaefer et al., 2001). The map shown in Fig. 5 is from a single animal.

3.4 Relationship of CT1 glomeruli to defined glomeruli

The highly restricted targeting of CT1-expressing axons in the olfactory bulb raised

the possibility that there was a spatial relationship between CT1 positive glomeruli

and known odorant receptor glomeruli. The M72 odorant receptor axons project to a

glomerulus located on the dorsal-lateral surface and a glomerulus located on the

dorsal-medial surface of each olfactory bulb (Potter et al., 2001). We initially

identified the localisation of the M72 glomeruli in sections of olfactory bulbs from

M72-GFP mice (Potter et al., 2001) using fluorescence microscopy. In sections

adjacent to those in which fluorescently stained M72-GFP glomeruli were located, we

detected CT1-positive glomeruli (arrows in Fig. 6A-D). Of particular interest was that

these CT1 glomeruli appeared to be isolated from other CT1-glomeruli and instead

were surrounded by glomeruli that did not express CT1 (Fig. 6B, D). Double label

fluorescent immunohistochemistry was then used to visualise both the M72

glomerulus and the CT1 glomeruli simultaneously in the same section. This analysis

revealed that the M72 glomeruli on both the lateral (Fig. 6E-G) and the medial (Fig.

6J-L) surfaces did not express the CT1 carbohydrate. One to two glomeruli that were

in close proximity to the lateral (Fig. 6H-I) or medial (Fig. 6M-N) M72 glomeruli

expressed CT1, while other adjacent glomeruli exhibited no CT1 expression.

The ventral distribution of isolated CT1 glomeruli in the olfactory bulb led us to

examine the relationship between the CT1 glomeruli and the P2 odorant receptor

glomeruli in P2-lacZ mice (Mombaerts, 1996). Sections adjacent to the P2 glomerulus

revealed the presence of at least one prominently stained CT1 glomeruli on each of

the medial (Fig. 7A-B) and lateral (Fig. 7C-D) surfaces of the olfactory bulb. Double

label fluorescence immunohistochemistry for ß-galactosidase (lacZ gene product) and

CT1 revealed that neither the lateral (Fig. 7E-G) nor the medial (Fig. 7I-K) P2 odorant receptor glomeruli expressed CT1. However on both surfaces of the bulb there was always a CT1 positive glomerulus apposed to the P2 glomerulus, although their relative positions were variable (Fig. 7H, L). Thus, we have revealed here a new level of precision in the local topography of glomeruli. Since P2 axons and CT1 axons always terminate in neighbouring glomeruli it raises the idea that these glomeruli share either significant functional characteristics or developmental origins.

3.5 Putative role of CT1 in axon targeting

We have previously generated a transgenic line of mice (BGAT-Tg) in which the blood group A (BGA) carbohydrate is ectopically expressed by all primary olfactory axons. In these mice, the targeting of M72 odorant receptor axons in the main olfactory bulb and of the vomeronasal axons in the accessory olfactory bulb was perturbed (St John et al., 2006). We had speculated that the ectopic expression of BGA could disrupt the expression of other carbohydrates. Ectopic expression of the BGA glycosyltransferase could have competitively inhibited the production of other carbohydrates synthesised from the same precursor substrate. However, at the time we were unable to detect changes in any candidate carbohydrates that could account for the guidance defects in M72 axons. Since the CT1 carbohydrate and the BGA carbohydrate are both synthesised from the common precursor Galß(1-4)GlcNAc (King, 1994; Lefrancois and Bevan, 1985) we postulated that ectopic expression of BGA transferase may have perturbed expression of CT1.

We therefore examined the expression of CT1 and BGA in wild type and BGAT-Tg mice. In wild type mice, CT1 was expressed in a subset of neurons in the main

olfactory epithelium (Fig. 8A) whereas, as previously reported (St John et al., 2006), BGA was not expressed (Fig. 8B). In marked contrast, CT1 expression was completely lost (Fig. 8C) while BGA was instead ectopically expressed by all olfactory sensory neurons (Fig. 8D) in BGAT-Tg mice. Similar results were observed in the vomeronasal organ of these transgenic mice (data not shown). Unfortunately, in the absence of an independent marker of CT1 axons, it was not possible to analyse the targeting of these axons in the BGAT-Tg mice. Nonetheless, given that CT1 axons normally target glomeruli adjacent to the M72 glomerulus, it raises the possibility that loss-of-CT1 function may have perturbed the sorting of CT1 and M72 axons which would account for the local axon guidance defects displayed by M72 axons.

4. Discussion

In the present study we have shown that the CT1 carbohydrate is mosaically expressed by ~20% of primary olfactory neurons throughout the olfactory epithelium. These CT1-positive neurons project axons to glomeruli that are distributed in several regions of the olfactory bulb but predominantly on the medial-dorsal surfaces. We have previously proposed (Key and St John, 2002) that axon guidance in the primary olfactory system depends on a hierarchy of cues that act at different spatiotemporal positions in the olfactory nerve pathway. We envisaged the presence of globally expressed cues, cues specific for various domains or regions of the nasal cavity and cues that are mosaically expressed both within and across these domains that are involved in the gross sorting of axons. The CT1 carbohydrate is a strong candidate for a sorting cue since not only is it mosaically expressed by primary olfactory neurons but the axons of these CT1 expressing neurons sort, converge and terminate in a subpopulation of glomeruli in the olfactory bulb.

The initial observation of the convergence of axons expressing the same odorant receptor to two topographically constant glomeruli in each olfactory bulb raised the idea that the odorant receptor proteins were themselves mediating axon guidance (Ressler et al., 1994; Vassar et al., 1994). Because CT1 is expressed by ~20% of olfactory neurons and a large number of glomeruli it is more difficult to assess the nature of the topographical organisation of these neurons, particularly when there is some degree of local variability in the relative final position of each glomerulus (Lipscomb et al., 2003; Schaefer et al., 2001; Strotmann et al., 2000). If the CT1 carbohydrate was expressed non-specifically by neurons in the olfactory epithelium

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then it would be expected that the axons of these CT1-expressing neurons would terminate in glomeruli which were not topographically fixed, either in the left and right olfactory bulbs of the same animal or in bulbs from different animals. Several observations here indicate that this is not the case, and that CT1 expression must be tightly controlled and probably linked somehow to the expression of certain odorant receptor genes. CT1 axons clearly terminate in a subpopulation of glomeruli. These glomeruli are restricted to specific regions on the surface of the olfactory bulb. While they are more concentrated on the dorsomedial surface of the bulb, there are patches located in spatially defined regions of the ventromedial and ventrolateral olfactory bulb which are totally devoid of CT1 glomeruli. Within the dorsomedial olfactory bulb, the CT1 glomeruli are typically grouped as discrete clusters of 2-4 apposing glomeruli. Despite technical limitations associated with obtaining sections in the same coronal plane it was possible to identify certain glomeruli which appeared to be in relatively constant positions in separate olfactory bulbs. These glomeruli could be identified both on the medial and lateral surfaces of the olfactory bulb. Thus, CT1 glomeruli are not stochastically distributed across the surface of the olfactory bulb. The axons of CT1 expressing primary olfactory neurons are clearly converging and form specific glomeruli within topographically defined regions. Moreover, within these regions the CT1 glomeruli are spatially restricted.

Notwithstanding the above results, the strongest evidence that refutes the idea that CT1 is expressed non-specifically by neurons in the olfactory epithelium is the observation that CT1 was never expressed by olfactory neurons expressing either the P2 or the M72 odorant receptors. More importantly was the observation that a CT1 glomerulus was always apposed directly to the M72 glomerulus on both the lateral

and medial surfaces of the olfactory bulb. Very little is understood about the spatial arrangements of glomeruli at the level of resolution of individual glomeruli. Using gene targeting approaches it has been shown that two genetically distinct populations of axons expressing different members of the OR37 odorant receptor gene family typically terminated in glomeruli that were directly apposed to each other in 80% of cases (Strotmann et al., 2000). However it is not the case that all families of related receptor genes target apposing glomeruli. The axons expressing the P2, P3 and P4 receptor genes terminate closely together but not in adjacent glomeruli (Feinstein et al., 2004). The closely apposed localisation of the CT1 and P2 glomeruli appears similar to the spatial relationship of the OR37 genes while the separated CT1 and M72 glomeruli are similar to that observed for the P2 subfamily. Taken together, the results indicate that the CT1 carbohydrate is most likely expressed by primary olfactory neurons which also consistently and specifically express a number of different odorant receptor genes. While these genes remain presently unknown it is likely that the CT1 expressing subpopulation incorporates up to ~200 different odorant receptor gene subpopulations. Ultimately, it is our goal to identify which specific odorant receptor neurons express the CT1 antigens. This would further verify that CT1 is expressed by select subpopulations of neurons and would provide an insight into the specific role of CT1 for particular odorant receptor neurons. In particular, we would like to map the distribution of different carbohydrates with specific odorant receptor glomeruli in order to provide a map of carbohydrates relevant to individual odorant receptor species.

We have taken advantage of a transgenic line of mice (BGAT-Tg) we have previously generated which mis-expresses the glycosyltransferase responsible for synthesising the A blood group in all primary olfactory neurons (St John et al., 2006). Our

previously published analyses of axon targeting in BGAT-Tg mice revealed that a small proportion of M72 odorant receptor axons inappropriately projected to glomeruli close to the M72 glomerulus (St John et al., 2006). In addition, vomeronasal axons, particularly those that targeted the caudal region of the accessory olfactory bulb, over-projected into the external plexiform layer of BGAT-Tg mice. We have previously speculated that the ectopic expression of BGA transferase perturbs the expression of other carbohydrates through competitive use of common precursors but we had been unable to identify any carbohydrates with altered expression patterns that would explain the observed axon guidance defects. We have now shown that the expression of CT1 is completely down-regulated in BGAT-Tg mice with no detectable expression of CT1 by main or accessory olfactory neurons. Unfortunately, the loss of CT1 prevented us from performing further analyses on the fate of the axons that normally would express this carbohydrate. While these results do no provide direct evidence that CT1 alone contributes to olfactory axon guidance, the expression of CT1 on subpopulations of main and accessory olfactory axons in the regions where axon guidance defects occur in BGAT-Tg mice is consistent with a role of CT1 in axon guidance. Our previous analyses of the mis-targeting of M72 odorant receptor axons in the BGAT-Tg mice revealed that a small proportion of M72 axons inappropriately entered neighbouring glomeruli particularly those immediately adjacent to the M72 glomerulus (St John et al., 2006). As we have now shown that CT1 is expressed by axons that target a glomerulus adjacent to the M72 glomeruli we propose that loss-of-CT1 contributes to an altered glycocode which perturbs the sorting of M72 and the now CT1 negative axons in the vicinity of the M72 glomerulus (Fig. 9). Similarly, we have also shown that during development CT1 is expressed by a small subpopulation of vomeronasal axons that project to the caudal

region of the accessory olfactory bulb. In the BGAT-Tg mice we had previously found the majority of axons that over-projected into the external plexiform layer emerged from the caudal region of the accessory olfactory bulb (St John et al., 2006). Thus, these results are consistent with a role for CT1 in olfactory axon guidance. It remains to be determined whether cell surface carbohydrates such as CT1 play a role in axon guidance during regeneration and whether the expression of carbohydrates is dependent on neural activity. It would be informative to determine if carbohydrates continue to be selectively expressed during widespread regeneration such as that stimulated by chemical destruction of the olfactory epithelium by dichlobenil (St John and Key, 2003), methimazole (Chehrehasa et al., 2010) or $ZnSO_4$ (Harding et al., 1978). Moreover such analyses would also determine if these carbohydrates contribute to the successful topographic reinnervation of glomeruli. In addition, by occluding the naris (Brunjes, 1994) it would be possible to begin to understand the role of neuronal activity on the expression of carbohydrates in the olfactory epithelium. Future experiments will examine these potential roles of CT1 and other cell surface carbohydrates.

5. Conclusion

Our results support the hypothesis that a glycocode (St John and Key, 2005) contributes to the establishment of the olfactory topographic map. We envisage that broadly expressed carbohydrates define the regional fasciculation of large subpopulations of axons. More restricted expression of carbohydrates, such as the DBA ligands which are present on ~30% of axons (Key and Akeson, 1993; Lipscomb et al., 2002; Lipscomb et al., 2003), might subdivide the large bundles into smaller discrete fascicles. Further refinement and subdivision is then mediated by

carbohydrates such as the CT1 carbohydrate which are expressed on ~20% of axons. Together, the differential combination of carbohydrates enables individual odorant receptor axons to be uniquely identified. Overall these observations support the postulate that olfactory axon guidance is achieved through a combination of axon guidance signals, and that cell surface carbohydrates contribute by providing identity to subpopulations of axons.

Acknowledgements

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Figure Legends

Figure 1. CT1 is expressed by primary olfactory neurons. Panels show coronal cryostat sections through the olfactory epithelium and olfactory bulbs, with dorsal to the top. (A) In E18 OMP-ZsGreen transgenic mice that expressed ZsGreen in primary olfactory neurons (green), a few immature neurons that expressed low levels of ZsGreen were strongly immunolabelled with anti-CT1 antibodies (red, arrow). (B) Higher power view of panel shown in A. The axon of the CT1-positive neuron is indicated by the double-headed arrow. (C-E) In P7 animals, CT1-positive neurons (red) and their axons (double-headed arrow) were more numerous. OMP-ZsGreen neurons are shown in D and the double-label image of C and D is shown in panel E. (F) In the adult, a proportion of neurons were labelled by CT1 antibodies using DAB immunohistochemistry. (G-H) In postnatal day 4 animals, CT1-positive neurons were present in all regions of the olfactory epithelium in the rostral (G) and caudal (H) portions of the epithelium. CT1 axons projected to glomeruli in regions throughout the olfactory bulb. CT1 neurons in the medial epithelium (arrows) were present at a higher density compared to CT1 neurons in the lateral epithelium (arrow with tail). NC: nasal cavity: lp: lamina propria; ob: olfactory bulb; oe: olfactory epithelium, s: septum. Scale bar is 50 µm in A, F, 25 µm in B-E, 300 µm in G-H.

Figure 2. Expression of CT1 in the olfactory bulb. Panels show coronal cryostat sections through the olfactory bulbs of P7 animals stained with diaminobenzidine immunohistochemistry, with dorsal to the top and medial to the left. (A-C) In sections from the rostral, central and caudal olfactory bulbs, CT1 was expressed in numerous glomeruli (arrowheads) along the medial surface (A) as well as scattered glomeruli in the dorsal and ventral surfaces (B-C). Isolated single glomeruli were also stained

(unfilled arrowhead in C). (D-F) Higher power images of glomeruli in the dorsal (D) and ventral (E) regions demonstrate the different levels of CT1 expression found within the olfactory bulb. Glomeruli expressing high levels of CT1 (arrow in E, F) were adjacent to glomeruli that expressed considerably less CT1 (arrowhead in E, F), while glomeruli which were completely negative for CT1 expression (dashed circles in F) were in close proximity. (G-H) Other examples of the differential expression of CT1. Some glomeruli expressed high levels (arrow), while others expressed lower levels (arrowhead) and adjacent glomeruli were completely negative for CT1. ML, mitral cell layer; NC, nasal cavity; NFL, nerve fibre layer; OE, olfactory epithelium. Scale bar is 600 µm in A-C; 50 µm in D-F; 75 µm in G-H.

Figure 3. Axons that express CT1 project to glomeruli in topographically fixed positions. Panels show coronal sections of the olfactory bulb of P7 animals with dorsal to the top. CT1 immunostaining of the left olfactory bulb is shown in A,D,G; the right olfactory bulb is shown in B, E, H; DBA lectin staining is shown in C, F, I. Immunostaining for CT1 in sections from the left and right olfactory bulbs in the equivalent rostro-caudal plane revealed that axons that express CT1 project to glomeruli (arrows) in clusters that were in topographically fixed positions; compare A-B, D-E, G-H. Isolated single glomeruli were also stained (e.g. arrows with tail in A,B). The DBA stained glomeruli were more numerous but were in topographically similar positions (C, F, I); sections were stained with haemotoxylin. (J-L) CT1 was expressed by glomeruli that also expressed DBA (arrow) whereas other glomeruli that expressed DBA (dashed oval) did not express CT1. Arrowheads show blood vessels that were immunostained by CT1. GL, glomerular layer; ML, mitral cell layer; NFL, nerve fibre layer. Scale bar is 500 µm in A-I; 50 µm in J-L.

Figure 4. CT1 is expressed by a subpopulation of vomeronasal neurons in early development. (A-D) In coronal sections through the vomeronasal organ of P7 OMP-ZsGreen mice (A), a small number of neurons in the basal region of the epithelium expressed CT1 (arrows in B); asterisk shows artefact. Double label with OMP-ZsGreen neurons is shown in C. A higher power view of the neurons in the ventral VNO is shown in D with cell body (arrow) and dendrites (double-headed arrow) being labelled by CT1 antibodies. (E) In a coronal section of the caudal region of a P7 accessory olfactory bulb which was immunostained for CT1 using diaminobenzidine, CT1 was strongly expressed in some glomeruli (arrow) and weakly expressed in others (arrowhead); medial is to the left. (F) In coronal sections of the adult accessory olfactory bulb (dashed outline), CT1 was no longer expressed; arrow points to a blood vessel; medial is to the left. (G) In contrast, staining of the same section using the lectin *Dolichos biflorus* agglutinin labelled all vomeronasal axons. Scale bar is 270 µm in A; 50 µm in B-C, E-G; 15 µm in D.

Figure 5. Schematic showing an example of the location of CT1-positive glomeruli from one olfactory bulb of a single postnatal day 7 animal. The olfactory bulb on the left was divided along the dorsal-ventral plane and then laid out flat in a butterfly preparation as indicated on the right. CT1-expressing glomeruli are shown with strong expression (black dots) or weak expression (grey dots). Regions where glomeruli did not express CT1 are represented by the grey patches. Blue dots represent P2 odorant receptor glomeruli; green dots represent M72 odorant receptor glomeruli. The dotted line represents the mid point along the dorsal-ventral axis.

Figure 6. CT1 is not expressed in M72 glomeruli. Panels are coronal sections of the olfactory bulb with lateral to the left and dorsal to the top. Panels in A-D show diaminobenzidine staining of CT1 in postnatal day 7 animals. (A-B) On the lateral surface of the olfactory bulb, a few CT1-positive glomeruli were present close to the position typical of the lateral M72 glomerulus; higher power of A is shown in B. (C-D) On the medial surface, a few isolated CT1-positive glomeruli were present close to the position typical of the medial M72 glomerulus; higher power view of C is shown in D. Panels E-N show coronal sections of the olfactory bulb of adult M72-GFP mice with lateral to the left and dorsal to the top. (E-G) The lateral M72-GFP glomerulus (green) did not express CT1, but had adjacent glomeruli that expressed CT1 (red). Arrow with tail in E and G shows a glomerulus that was negative for CT1. (H-I) Other examples showing that the lateral M72 glomerulus (green) did not express CT1 while surrounding glomeruli did. (J-L) The medial M72 glomerulus (green) did not express CT1, but adjacent glomeruli expressed high levels of CT1 (arrowhead) and low levels of CT1 (double-headed arrows). Section is stained with DAPI (blue). (M-N) Other examples showing that the M72-GFP glomerulus did not express CT1 while adjacent glomeruli did. Arrowhead in N shows a glomerulus that expressed high levels of CT1 and the double-headed arrow shows a glomerulus that expressed low levels of CT1. In all five animals that were examined, and in both the left and right olfactory bulbs, the CT1 glomeruli with strongest expression were within 2-3 glomerular widths of the lateral and medial M72 glomeruli. EPL, external plexiform layer; GL, glomerular layer; NFL, nerve fibre layer. Scale bar is 125 μm in A, C; 50 μm in B, D; 60 μm in E-N.

Figure 7. CT1 glomeruli are adjacent to P2 glomeruli. Panels are coronal sections with lateral to the left and medial to the right; panels in A-D show diaminobenzidine staining of CT1 in postnatal day 7 mice. (A-B) On the lateral-ventral surface of olfactory bulb, CT1 was expressed by a few isolated glomeruli close to the position typical of the lateral P2 odorant receptor glomerulus (arrow); higher power of A is shown in B. (arrow). (C-D) On the medial-ventral surface of olfactory bulb, CT1 was expressed in glomeruli close to the position typical of the medial P2 odorant receptor glomerulus (arrow); higher power of C is shown in D. (E-G) Immunolabelling of sections of P2-lacZ adult mice revealed that the lateral P2 glomerulus (green, arrow in E) was adjacent to glomeruli that expressed CT1 (double-headed arrows in F). Double-labelled image is shown in G; arrowhead in G points to an autofluorescent blood vessel. (H) Another example of a CT1 glomerulus adjacent to the P2 glomerulus on the lateral surface of the olfactory bulb. (I-K) The medial P2 odorant receptor glomerulus (green, arrow) was adjacent to glomeruli that expressed the CT1 carbohydrate (red, double-headed arrows). Arrowhead in K points to an autofluorescent blood vessel. (L) Another example of the medial P2 glomerulus with two adjacent CT1 glomeruli. GL, glomerular layer; NFL, nerve fibre layer. Scale bar is 350 µm in A, C; 140 µm in B, D; 50 µm in E-L.

Figure 8. CT1 is not expressed in BGAT-Tg mice. (A) In the olfactory epithelium (OE) of wild type mice, CT1 distinctly labelled a subset of neurons. (B) In contrast, no neurons were immunolabelled with Blood Group A antibodies. (C) In BGAT-Tg mice, CT1 was not expressed by primary olfactory neurons; (D) whereas BGA was strongly expressed by all neurons. LP, lamina propria; NC, nasal cavity; OE, olfactory epithelium. Scale bar is 50 µm.

Figure 9. CT1 contributes to axon sorting. Schematic diagrams of primary olfactory axons targeting glomeruli within the olfactory bulb. (A) In wild type mice, axons that express the CT1 carbohydrate (red outline) project to a glomerulus that is adjacent to the glomerulus in which M72 odorant receptor axons (green) terminate. (B) In BGAT-Tg mice, the CT1 carbohydrate is not expressed by any axons and instead BGA carbohydrate (blue outline) is expressed by all axons. While many of the M72 odorant receptor axons (green) target the M72 glomerulus a small proportion of M72 axons inappropriately enter neighbouring glomeruli (arrows).

Figure 1
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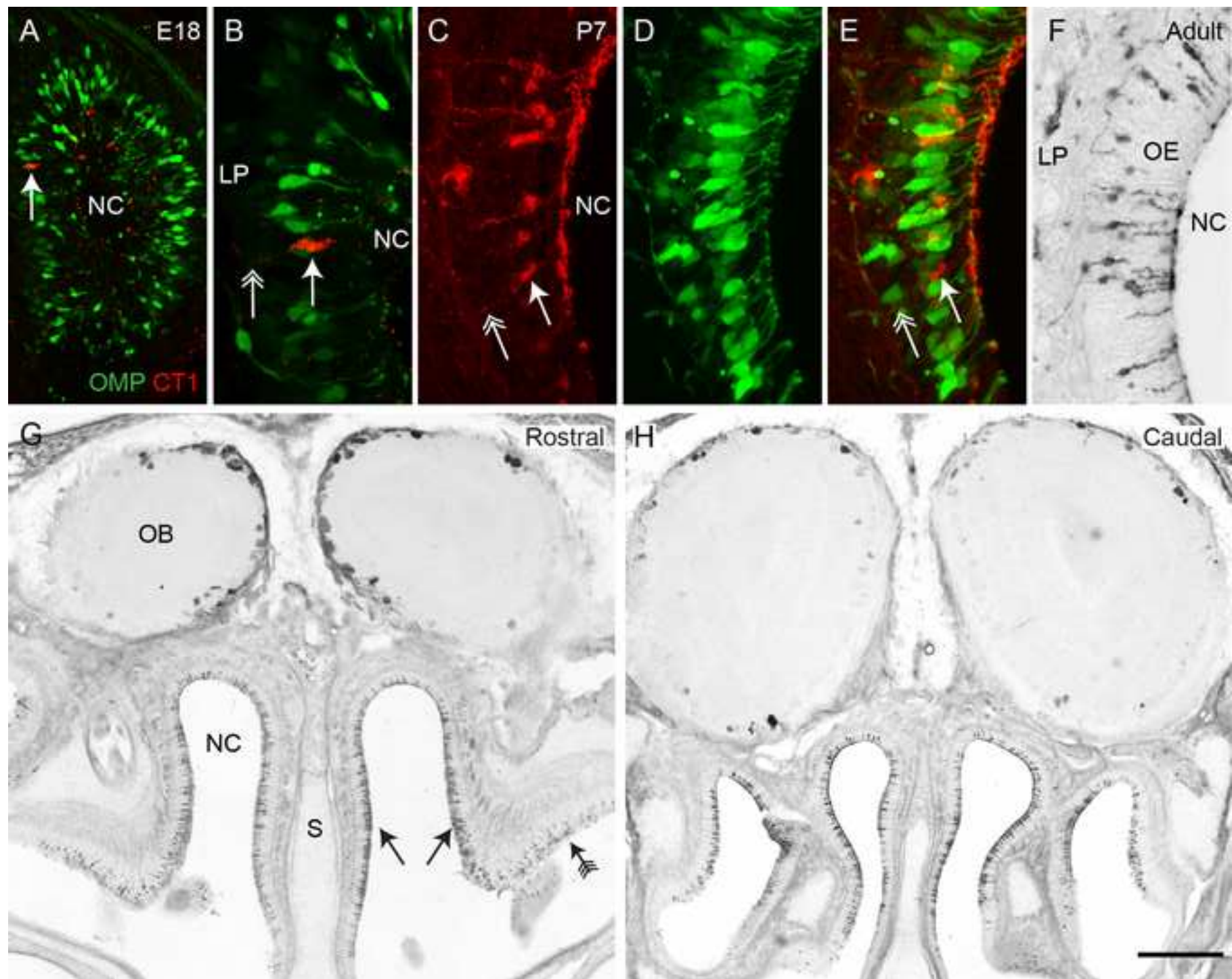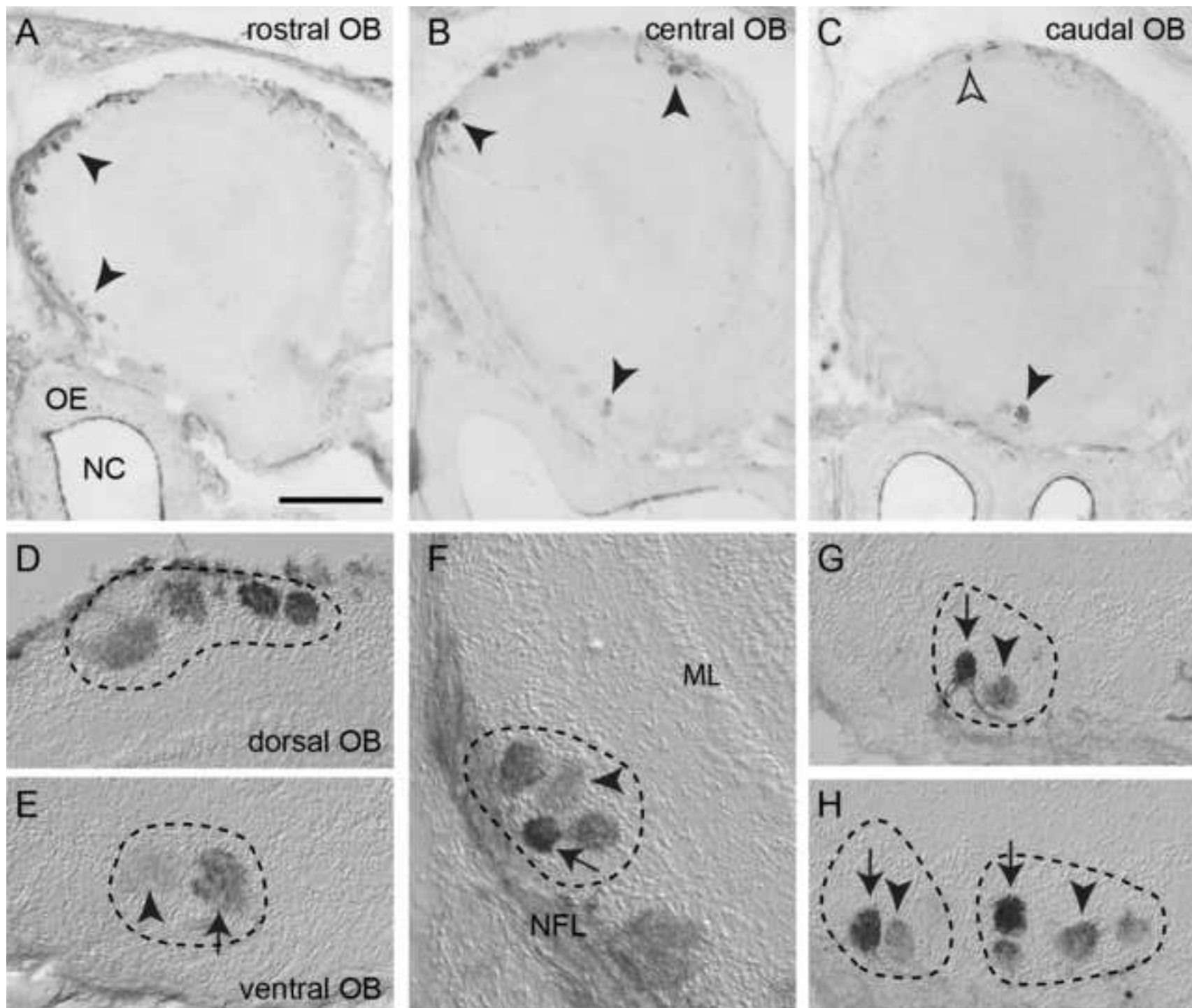
Figure 3
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A — CT1 left OB - rostral (GL)
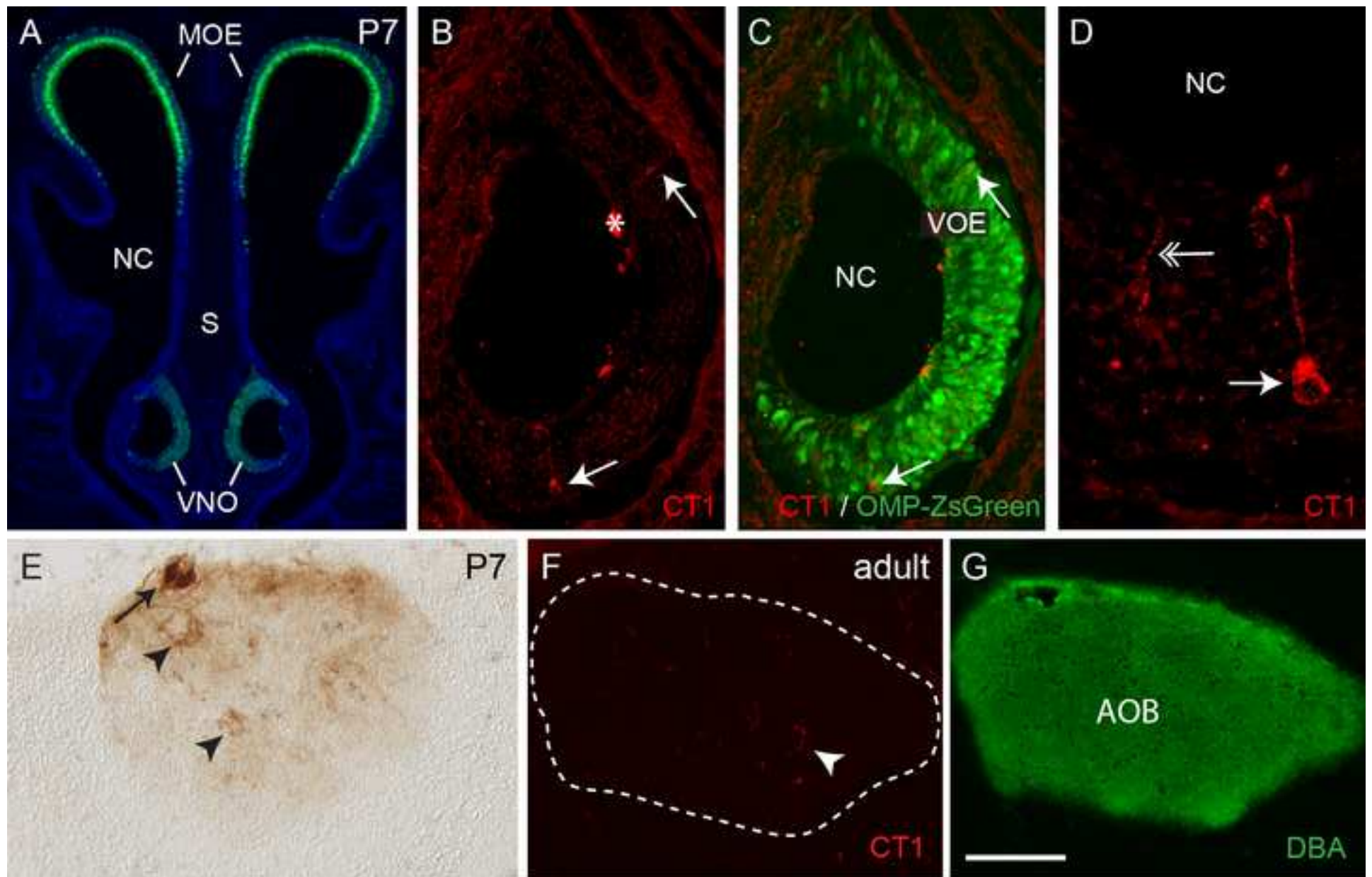
B — CT1 right OB - rostral

C — DBA - rostral

D — CT1 left OB - central (ML, GL, NFL)

E — CT1 right OB - central

F — DBA - central

G — CT1 left OB - caudal

H — CT1 right OB - caudal

I — DBA - caudal

J — CT1

K — DBA

L — DBA CT1
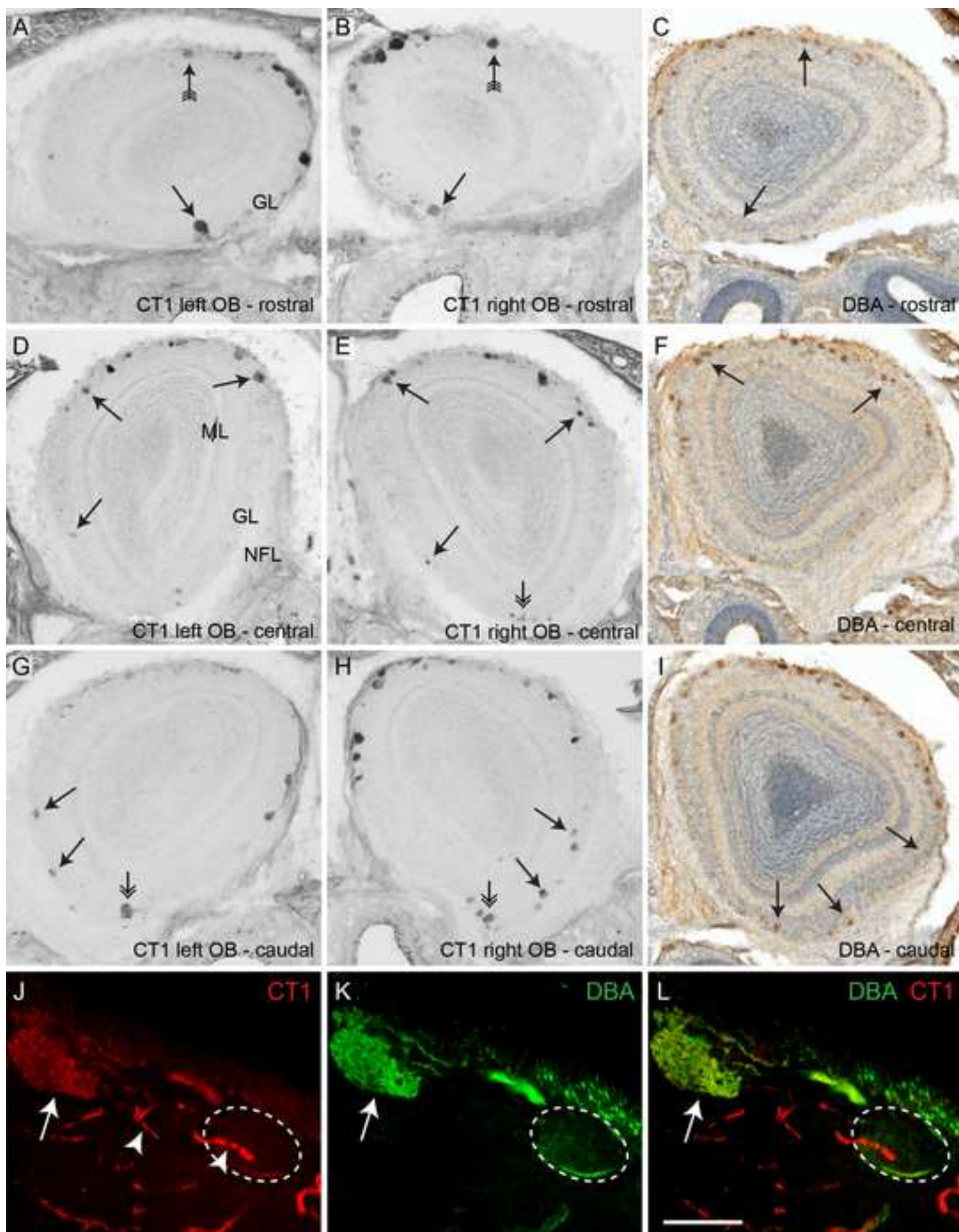
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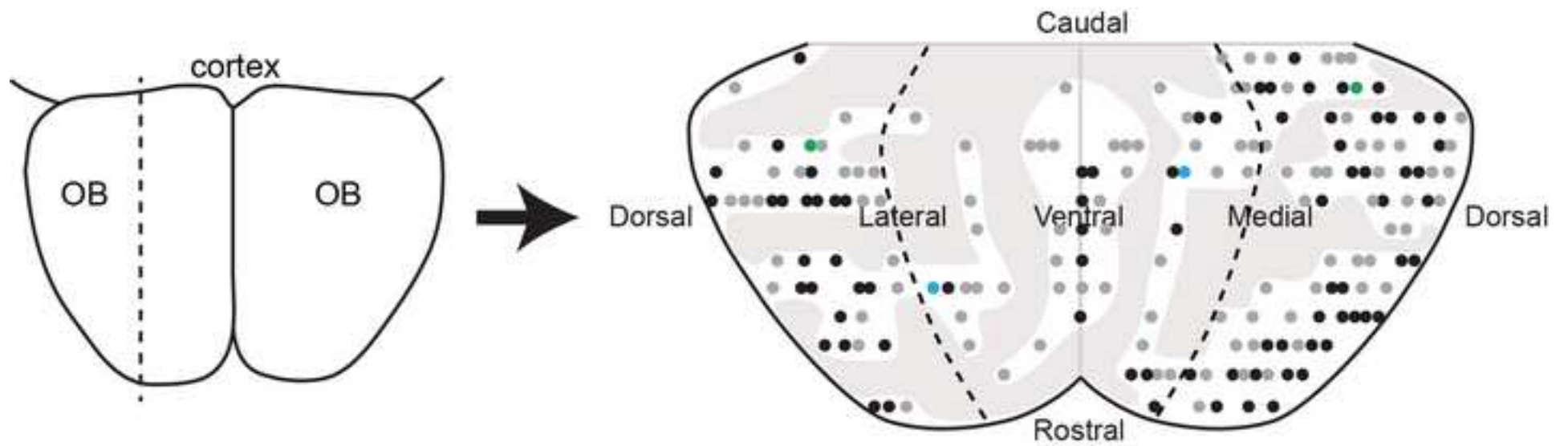
Figure 6
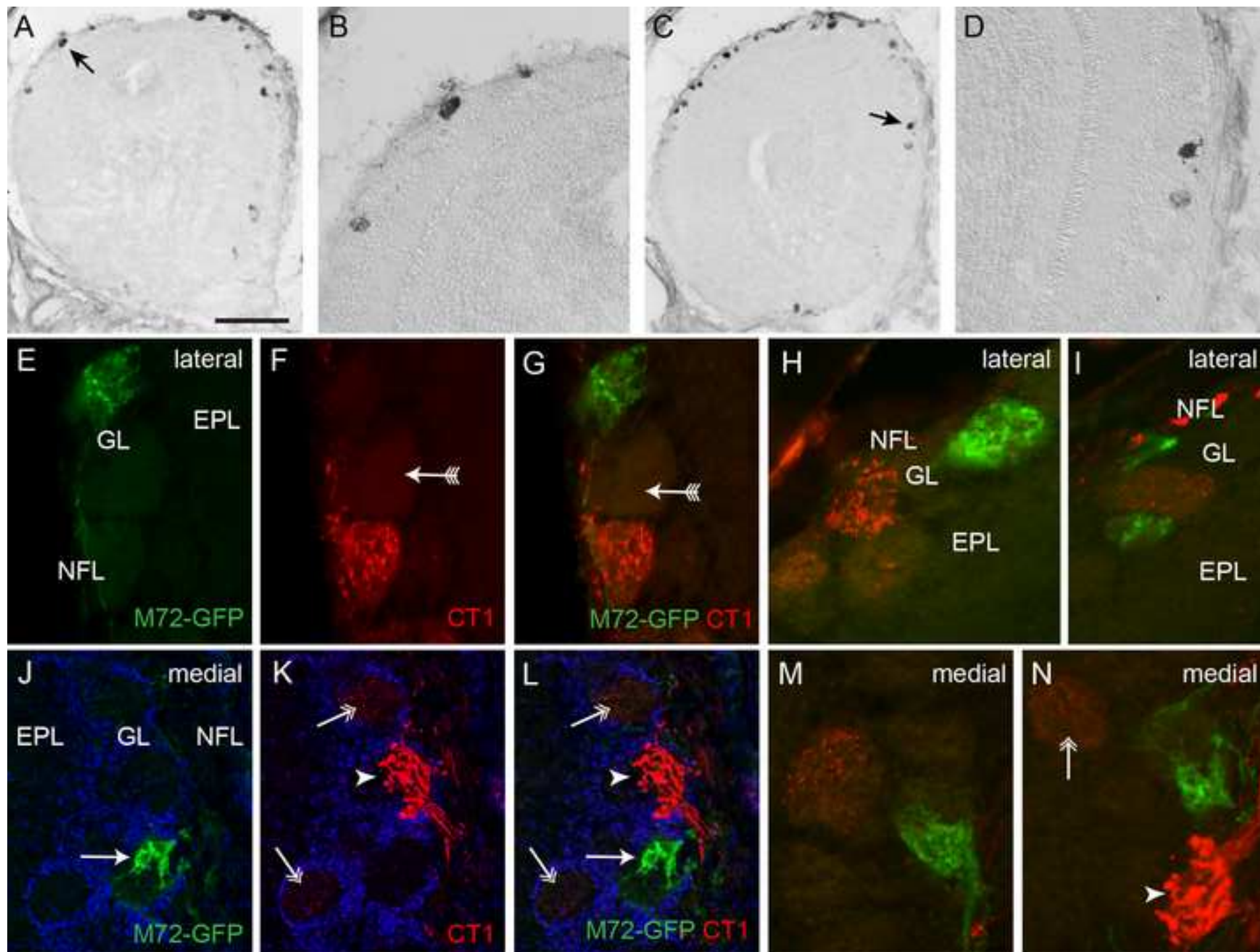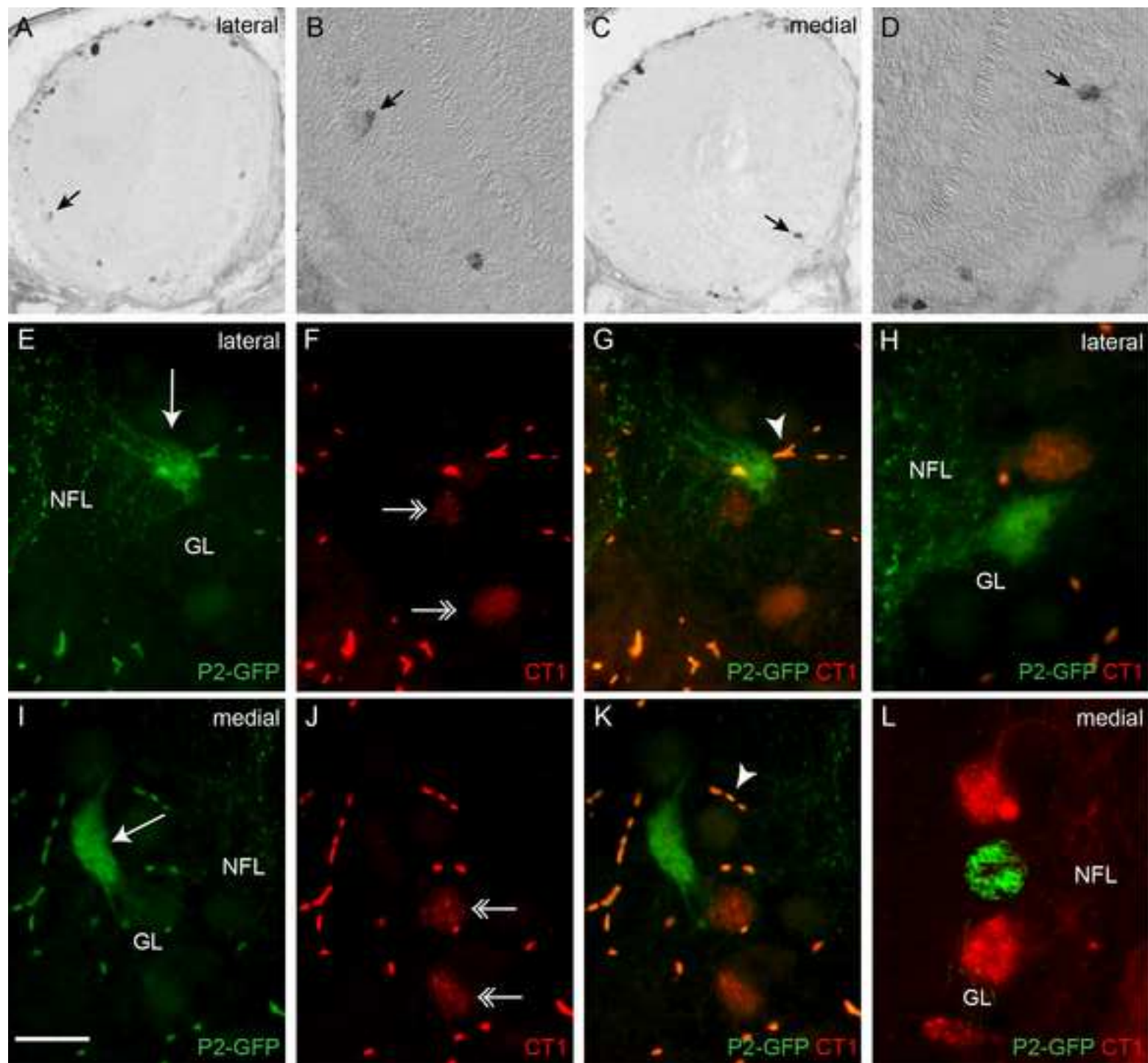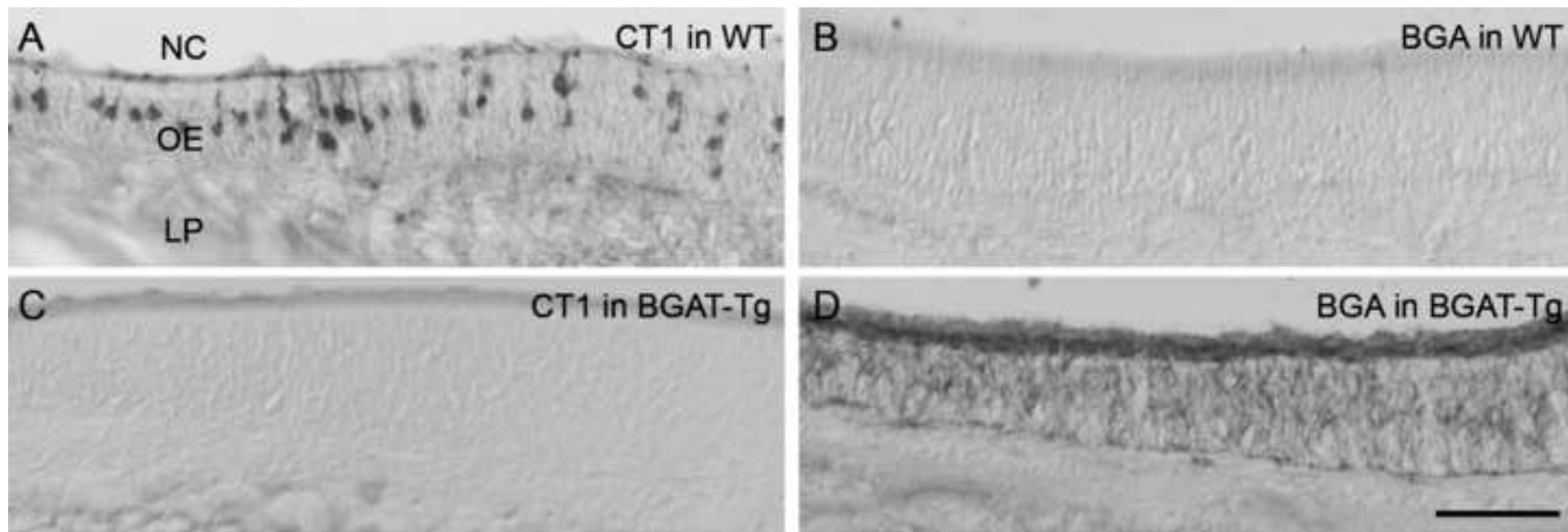**Click here to download high resolution image**

A — NC, OE, LP — CT1 in WT	B — BGA in WT
C — CT1 in BGAT-Tg	D — BGA in BGAT-Tg

A wild type mice

CT1 carbohydrate
M72 axons

B BGAT-Tg mice

CT1 carbohydrate not expressed
BGA carbohydrate
M72 axons mistarget