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The cell surface carbohydrate blood group A regulates the selective fasciculation of regenerating accessory olfactory axons

by

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Abstract

Cell surface carbohydrates are differentially expressed by discrete subpopulations of primary sensory axons in the mammalian main and accessory olfactory systems. It has been proposed that these carbohydrates provide a glycode which mediates the sorting of these sensory axons as they project from the olfactory neuroepithelium to their central targets in the main and accessory olfactory bulbs during development. As the differential expression of cell surface carbohydrates on olfactory axons persists in the adult we have now investigated their role during regeneration. We have recently generated a line of transgenic mice, BGAT-Tg, that mis-express the blood group A (BGA) carbohydrate on all primary olfactory axons rather than just on accessory olfactory axons as in wild-type mice. Following unilateral bulbectomy, accessory and main olfactory axons regenerate and grow into the frontal cortex where they fill the cavity which remains after the olfactory bulb ablation. In wild-type mice, the regenerating BGA-expressing accessory olfactory axons selectively aggregated with each other in large bundles but clearly separated from the BGA-negative main olfactory axons. In contrast, in the BGAT-Tg transgenic mice in which all main and accessory axons express the BGA carbohydrate, the accessory olfactory axons failed to correctly separate from the main olfactory axons. Instead, these axons formed numerous small bundles interspersed with main olfactory axons. These data provide strong evidence that the restricted expression of BGA is in part responsible for the selective segregation of accessory olfactory axons.

Classification terms

Scope: Nervous System Development, Regeneration and Aging

Keywords: vomeronasal, neuron, regeneration, targeting, guidance, glomerulus
Abbreviations

BGA: blood group A

OMP: olfactory marker protein

VNO: vomeronasal organ
**Introduction**

In the mammalian olfactory system, primary olfactory neurons expressing the same odorant receptor are scattered throughout restricted zones in the nasal cavity (Vassar et al., 1993; Ressler et al., 1994; Strotmann et al., 1994a; Strotmann et al., 1994b; Miyamichi et al., 2005; Tsuboi et al., 2006). In the mouse, each primary olfactory neuron within the olfactory epithelium lining the nasal cavity expresses one of a possible 1300 different odorant receptors. The axons of these neurons sort out and converge as they project to each olfactory bulb, where they terminate in approximately two topographically-fixed glomeruli (Ressler et al., 1994; Vassar et al., 1994; Nagao et al., 2000). At present, the sorting of these axons en route to their target is known to involve the coordinated expression of a limited suite of guidance molecules, including at the most restricted level, the odorant receptors which mediate the final topographic position of glomeruli (Mombaerts et al., 1996; Wang et al., 1998). However, it remains unclear what the mechanism is that controls the differential segregation of primary sensory olfactory axons prior to final targeting.

Cell surface carbohydrates have intriguing expression patterns on discrete subpopulations of primary sensory axons in both the main and accessory olfactory systems (Allen and Akeson, 1985; Key and Giorgi, 1986; Schwarting and Crandall, 1991; Schwarting et al., 1992; Dowsing et al., 1997; Pays and Schwarting, 2000; St John and Key, 2001; Lipscomb et al., 2002; Lipscomb et al., 2003; Salazar and Sanchez Quinteiro, 2003; St John and Key, 2005; St John et al., 2006). It has been proposed that the differential expression of carbohydrates may provide a glycocode which mediates the sorting out and selective segregation of these sensory axons (see review by (Key and St John, 2002)). While carbohydrates have been implicated in
regulating olfactory axon growth and fasciculation (Ichikawa et al., 1994; Storan et al., 2004; Henion et al., 2005; St John et al., 2006), the presence of other guidance cues and the spatial and temporal restraints on mixing are likely to have masked some of the role of the carbohydrates. Hence we postulated that if the potential spatial and temporal restraints were removed or reduced then the role of the cell surface carbohydrates may be revealed. To test this we have performed unilateral bullectomies to stimulate widespread axon regeneration in the absence of the normal spatial and temporal restraints; and then examined the segregation of vomeronasal (VNO) accessory and main olfactory axons in mice in which the normal complement of cell surface carbohydrates is perturbed. In wild-type mice, blood group A (BGA) is selectively expressed during early development only by a subpopulation of VNO axons that terminate in a unique caudal zone of the accessory olfactory bulb (St John et al., 2006), whereas in the adult BGA is expressed by all VNO axons. At no stage do the primary sensory olfactory axons of the main olfactory system express this carbohydrate epitope. In contrast, in BGA glycosyltransferase transgenic (BGAT-Tg) mice BGA is expressed by all sensory neurons of the main and accessory olfactory systems under the control of the olfactory marker protein (OMP) promoter both during embryonic development as well as into adulthood (St John et al., 2006). Thus, comparing the effect of bullectomy on axon segregation in both wild-type and BGAT-Tg mice provides an opportunity to determine the role of the restricted expression pattern of BGA in axon growth.

We have recently shown that within four weeks of neonatal bullectomy the VNO and main olfactory axons grow back into the cranial cavity and form an amorphous plexus of axons (Chehrehasa et al., 2006). In this plexus, the VNO axons were specifically
sorted from the olfactory axons and typically formed a large discrete bundle that was separate from the main olfactory axons. This occurred despite their close physical proximity to main olfactory axons suggesting that distinct cues were operating to regulate the selective bundling of VNO axons. However, we have now demonstrated that when unilateral bulbectomies were performed in BGAT-Tg mice in which the carbohydrate expression is perturbed, there were considerable deficits in the bundling of vomeronasal axons during regeneration. In these transgenic mice, the VNO axon fascicles failed to selectively bundle with each other and instead formed numerous isolated small fascicles that were intermingled with fascicles of main olfactory axons. These data provide strong evidence that the cell surface BGA carbohydrate is involved in the segregation of VNO axons in the regenerating olfactory system.
Results

Blood group A carbohydrate is expressed by VNO axons

During development of the accessory olfactory system, the vomeronasal (VNO) axons selectively express the blood group A (BGA) carbohydrate (St John et al., 2006). Anti-BGA immunohistochemistry of coronal sections of the head confirmed that the expression of BGA was restricted to VNO axons terminating in the accessory olfactory bulb in 4-week old wild-type mice (Fig. 1a). In contrast, main olfactory neurons in the olfactory neuroepithelium (double-headed arrow, Fig. 1c) and their axons that project to glomeruli in the main olfactory bulb do not express BGA (Fig. 1c). However, in transgenic mice in which the OMP promoter drives expression of the BGA glycosyltransferase (BGAT-Tg mice) (St John et al., 2006), all primary sensory neurons of the main and accessory olfactory systems expressed BGA (Fig. 1b,d). We have postulated that the ubiquitous expression of BGA by neurons throughout both the main and accessory olfactory systems would perturb the segregation or bundling of regenerating VNO axons. In order to test the role of BGA in regenerating VNO axons, we performed unilateral bulbectomies on postnatal day 4.5 wild-type and BGAT-Tg littermate mice and allowed the mice to recover for 4 weeks.

Altered fasciculation of VNO axons during regeneration in BGAT-Tg mice

Four weeks following bulbectomy, neurons in the VNO regenerated and projected axons towards the cranial cavity. Immunostaining with anti-KH10 antibodies, which have previously been shown to selectively label VNO neurons and their axons (Storan et al., 2004), revealed that there was no difference in the distribution of the regenerated neurons in the neuroepithelium of the VNO of either the BGAT-Tg or
wild-type littermate mice (Fig. 2a-b). Furthermore, neurons of the main olfactory neuroepithelium also regenerated (data not shown) as previously demonstrated (Chehrehasa et al., 2006).

Analysis of the regenerated accessory and main olfactory axons revealed that these axons had grown into the cavity previously occupied by the ablated olfactory bulb. Immunolabelling with anti-OMP antibodies, which labels all main and VNO axons, revealed that these regenerated axons were spread throughout an amorphous plexus (red, Fig. 2d,f-j). Double labelling of the AOB within the unoperated side of both wild-type and BGAT-Tg mice with anti-KH10 and anti-OMP antibodies demonstrated that KH10 selectively labelled only VNO axons that terminated in the AOB (Fig. 2c,e). In the operated side of wild-type mice, the regenerated VNO axons typically formed a single large bundle of adjoining fascicles (dotted line, Fig. 2d). This main bundle was predominantly located in the medio-dorsal portion of the axon plexus. In addition, a small number (1-3) of VNO axon fascicles/bundles were typically separated from, but remained close to, the main bundle (arrow, Fig. 2d).

In contrast, the BGAT-Tg mice displayed a considerably different pattern of regenerated VNO axons. Rather than form a prominent large bundle as observed in wild-type mice, the regenerated VNO axons formed a much smaller main bundle of axons (Fig. 2d, f-h) which was not restricted to the medio-dorsal region of the plexus. Instead, this bundle was found in various positions throughout the plexus in different animals. In addition, numerous small fascicles or bundles of VNO axons were dispersed throughout the remaining regions of the plexus populated by the main olfactory axons. In contrast to the wild-type mice, these small VNO axon bundles
were widely dispersed and clearly separated from the main bundle of VNO axons (arrows, Fig. 2f-h).

In order to quantify the degree of aggregation of the regenerated VNO axons, we measured the size of the largest bundle in each plexus (e.g. dashed line, Fig. 2d,f) and found that the VNO axons in BGAT-Tg mice formed bundles less than half the size of that in wild-type littermate mice (Fig. 3a; p<0.05). At high magnification it was clear that many small bundles or fascicles of VNO axons in the BGAT-Tg mice were also interspersed amongst fascicles of main olfactory axons (Fig. 2i-j). As the morphology of the axon fascicles was often irregular or indistinct we describe the VNO axons as being in bundles, which may consist of one or more fascicles. Using this approach we found that the VNO axons formed twice as many bundles in BGAT-Tg mice compared to their wild-type littermates (Fig. 3b; p<0.005). Examination of the VNO axons using anti-KH10 immunostaining in the VNO discounted the possibility that the perturbed bundling was due to differences in number of regenerated neurons given that the distribution of neurons was similar between BGAT-Tg and wild-type littermates (Fig. 2a-b). We also examined the number of axons that were present in the plexus as we considered it to be possible that while the neurons had regenerated, their axons may not have reached the plexus. We therefore measured the total area of axons by counting the labelled pixels present above the background threshold and found that the total area of axons in control (9.35x10^5 µm^2) and BGAT-Tg (7.92x10^5 µm^2) animals were not significantly different (p>0.05).
Discussion

The restricted expression of specific glycans by subpopulations of primary sensory neurons in the main and accessory olfactory systems has led us to propose a role for these molecules in axon fasciculation and bundling (Key and St John, 2002). We have previously shown in wild-type mice that regenerating main olfactory and VNO axons sort out on the basis of the specific cell surface glycoconjugates they express (Chehrehasa et al., 2006). This led us to hypothesize that expression of specific glycans by olfactory and VNO axons may play a role in segregation of VNO axons from olfactory axons during regeneration.

We have begun to address this hypothesis in the present study by examining the growth of olfactory axons which were stimulated to grow en masse into a bulbectomised brain lacking the normal spatiotemporal and physical restraints present during development. We have used a line of transgenic mice (BGAT-Tg) in which the blood group A (BGA) carbohydrate was ectopically expressed by all olfactory and VNO axons (St John et al., 2006); rather than the distinct expression only on VNO axons as seen in wild-type mice. Our results have clearly demonstrated that VNO axons failed to correctly aggregate together during regeneration in the BGAT-Tg mice. They instead formed numerous small bundles clearly separate from other VNO axon bundles and intermingled with bundles of main olfactory axons. One possibility here is that the BGA carbohydrate is acting as a sorting cue to selectively segregate axons. In Xenopus, we have previously demonstrated that a subpopulation of axons in the early developing brain express BGA and that perturbation of the BGA function resulted in axon mistargeting possibly through perturbed axon fasciculation.
Other carbohydrates have been implicated in regulating axon fasciculation. Inhibiting the binding of lactosamine carbohydrates in rat olfactory neuroepithelial explants reduced axon fasciculation (Storan et al., 2004) while VNO axons became defasciculated when the binding of glycoconjugates were blocked by lectins in vitro (Ichikawa et al., 1994). Cell surface carbohydrates can modulate the activity of adhesion molecules such as NCAM (Weinhold et al., 2005), suggesting that the mis-expression of BGA in the BGAT-Tg mice may have perturbed the activity of these molecules resulting in reduced bundling of VNO axons during regeneration.

We employed our bulbectomy model in which BGA was ectopically expressed so that the normal temporal and spatial restraints that are imposed during development were removed. This approach proved to be successful since we now observed the extensive intermingling of the regenerated VNO axon fascicles with fascicles of main olfactory axon that expressed BGA. These results indicate that BGA acts through an attractive mechanism, most likely mediated by galectin binding, which promotes the adhesion between fascicles of the VNO and main olfactory axons. However, several other molecules are known to be involved in VNO axon fasciculation and targeting and these are likely to have continued exerting their effects. During development, the VNO nerve grows along the medial surface of the main olfactory bulb in close proximity to the main olfactory axons, yet the VNO axons remain segregated from the main olfactory axons and do not enter the main olfactory bulb. Neuropilin-2 and Sema3F have been implicated in partially regulating the segregation since knockout of either molecule results in defasciculation of the VNO nerve and knockout of Sema3F also results in a small number of the VNO axons entering the main olfactory
bulb (Cloutier et al., 2002; Walz et al., 2002; Cloutier et al., 2004). These data have led to the proposal that the Sema3F acts in a surround repulsion mode to tightly fasciculate the VNO axons that express neuropilin-2 (Cloutier et al., 2004). During our extensive analysis of the development of the BGAT-Tg mice (St John et al., 2006) we did not observe VNO axons mixing with main olfactory axons. It is likely that the presence of repulsive molecules such as Sema3F may have provided sufficient repulsion to prevent the mixing of the different axon populations despite BGA being ectopically expressed by all axons in the BGAT-Tg mice. The cell adhesion molecule OCAM is expressed by VNO neurons located in the apical region of the VNO neuroepithelium and their axons project to the rostral AOB. In contrast, axons that do not express OCAM terminate in the caudal AOB (Alenius and Bohm, 1997; von Campenhausen et al., 1997; von Campenhausen and Mori, 2000). We have previously demonstrated that OCAM continues to be expressed by VNO axons following bullectomy (Chehrehasa et al., 2006) and thus OCAM is likely to at least partially regulate the adhesion of VNO axons in the BGAT-Tg mice. Other cues that are known to be involved in VNO axon targeting are ephrin-A5 which regulates the guidance of apical VNO axons to the rostral AOB (Knoll et al., 2001) as well as Robo-2 which regulates the guidance of basal VNO axons to the caudal AOB, although Robo-2 not considered to be crucial for axon fasciculation (Cloutier et al., 2004). However, bullectomy is likely to remove the source of the ephrin-A5 and Robo-2 binding partners that are expressed in the AOB and hence eliminate any resulting effects they may have had on axon fasciculation and subsequently on regenerating axons.
We consider that the fasciculation of axons is likely to involve a combination of cues, some of which would continue to exert their effects in our bullectomy model. Further, we propose that the cell surface carbohydrates such of BGA are strong candidates for regulating the selective segregation of subpopulations of carbohydrate-expressing axons and act via the carbohydrate binding proteins, galectins. Numerous galectins are known to be expressed in the nerve fibre layer of the olfactory system including galectin-1, galectin-3, galectin-4, galectin-7 and galectin-8 (Mahanthappa et al., 1994; Puche and Key, 1995; Puche et al., 1996; Tenne-Brown et al., 1998; St John and Key, 1999; Crandall et al., 2000; Storan et al., 2004). While these galectins bind BGA, they have different binding affinities which are therefore likely to lead to the selectively adhesion of different subpopulations of axons (Ideo et al., 2003; de Melo et al., 2007). However, when BGA was universally expressed by all regenerating axons in the bulbectomised mouse the ability of the galectins to discriminate between the subpopulations would have been considerably reduced, leading to inappropriate binding of fascicles of VNO and main olfactory axons within the regenerated plexus.

In summary, we have previously shown that BGA is involved in the targeting of VNO axons during development (St John et al., 2006) and now we have provided evidence that BGA mediates the selective segregation of VNO axons when they regenerate en masse. Thus it is clear from accumulating evidence that cell surface carbohydrates function to regulate the growth and segregation of main olfactory and VNO axons during development and regeneration.
Experimental Procedure

**BGAT transgenic mice**

Transgenic mice expressing the human blood group A glycosyltransferase (BGAT) in sensory neurons of the main and accessory olfactory systems were previously generated in our laboratory (St John et al., 2006). In these mice, referred to as BGAT-Tg, the full length (5.5kb) olfactory marker protein (OMP) promoter (Danciger et al., 1989) drives the expression of the human histo-blood group A transferase (Yamamoto and McNeill, 1996) in all primary and accessory olfactory neurons. Mice were genotyped by polymerase chain reaction (PCR) with genomic DNA isolated from mouse tails and the genotype was confirmed by immunohistochemistry for BGA.

**Surgical ablation of olfactory bulbs**

Unilateral bulbectomies were performed as previously described (Chehrehasa et al., 2006) on day P4.5 wild-type or hemizygous BGAT-Tg littermate mice. Briefly, pups were anaesthetised by placing them on ice for 8 min and a midline incision was made to expose the cranial bones. The bone directly over the olfactory bulb was removed using a surgical drill and the entire olfactory bulb was aspirated using a pulled glass pipette attached to a vacuum pump. The skin covering the site was sutured and the anti-inflammatory drug Flunix was administered via intramuscular injection (active ingredient flunixin, 1 mg/kg body weight; Parnell Laboratories, Australia). Animals were allowed to recover for 4 weeks. All procedures were carried out with the approval of, and in accordance with, the University of Queensland Animal Ethics Experimentation Committee and the Commonwealth Office of the Gene Technology Regulator.
Animal preparation

Four weeks after surgery the mice were killed by CO₂ asphyxiation. Heads were immersion fixed overnight at 4°C in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4). Following fixation, 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) and snap frozen by immersion in iso-pentane that had been cooled by liquid nitrogen.

Immunohistochemistry

Cryostat sections (30 µm) of the olfactory region and frontal cortex were cut, mounted on to gelatinized slides and stored at –20°C. Sections were incubated with 0.15% H₂O₂ in methanol for 5 min before being washed with 0.1M Tris buffered saline (TBS, pH 7.4) and 0.3% Triton-X-100 (TX-100) for 2 min. They were then incubated with 2% bovine serum albumin (BSA; Sigma Chemical Corporation) in 0.1M TBS with 0.3% Triton X-100 for 30 min at room temperature (RT). The sections were incubated overnight at 4°C with mouse monoclonal anti-BGA (neat; CSL Ltd., Australia) primary antibody in 2% BSA and 0.3% TX-100 in TBS. Sections were then washed with TBS/Triton-X-100 and incubated in biotinylated anti-mouse IgM (1:200; Sigma Chemical Co.) for 1 hr at RT, washed in TBS/Triton X-100 and then incubated with streptavidin Alexa Fluor 488 conjugate (1:200; Molecular probes, Eugene, OR) for 1 hr at RT for fluorescence imaging; alternatively sections were instead incubated with avidin-biotin-horseradish peroxidase (Vector Laboratories Inc) and staining was then visualized by reaction with 3,3’-diaminobenzidine (DAB) and hydrogen peroxide (H₂O₂) in TBS.
For double label immunohistochemistry, sections were incubated overnight at 4°C with goat polyclonal antiserum against OMP (1:1000) (Keller and Margolis, 1975) and mouse KH10 hybridomas developed by Thomas M. Jessel and Jane Dodd, Center for Neurobiology and Behavior, Columbia University, New York, NY, (obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA; (Dodd and Jessell, 1985). The sections were washed with TBS and TX-100 and then incubated in rabbit anti-goat immunoglobulin antibody conjugated to biotin (1:200; Vector Laboratories, Inc, CA, USA) for 1 hr at RT. The sections were then incubated for 1 hr at RT with mouse anti-IgM antibody conjugated to FITC (1:50; Jackson Immuno Research Laboratories, Inc, Baltimore) and streptavidin conjugated to texas red (1:50, Vector), before being washed and coverslipped with fluorescent mounting medium.

Images of the sections were captured either by confocal laser scanning microscopy (MRC 1024, Bio-Rad Laboratories Inc., Hercules, CA) using an Olympus BX60 microscope or with an Olympus BX51 compound fluorescence microscope. Serial optical sections were imaged at 5 µm intervals and were projected to give a two-dimensional reconstruction. Images were then compiled in Adobe Photoshop 7.0 and Adobe Illustrator 10.0 (Adobe Systems Incorporated).

Quantification of axon bundles
The number of KH10 positive fascicles was counted in the coronal section in which the largest bundle of fascicles was located within the regenerating plexus. The size of the largest bundle of VNO axons within the regenerating plexus was determined by summing the number of labelled pixels using the Image Pro 5.0 (Media Cybernetics, Inc., Silver Spring, MD). Statistical significance was assessed using a paired two-
tailed t-test. The number of KH10 positive fascicles bigger than 2.5 µm was counted in the coronal section of two sections in which the larger bundle of fascicles was located in the central region of the regenerated plexus. The total area covered by VNO axons was quantified using ImageJ; the threshold for all images was set at the same level and the number of pixels with staining above the threshold was quantified using the “analyse particles” function. Statistical significance was assessed using a paired two-tailed t-test.
Literature References


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Figure legends

Fig. 1. Blood group A is ectopically expressed by olfactory and vomeronasal (VNO) axons in BGAT-Tg mice. Panels are coronal sections through the olfactory bulb and main olfactory neuroepithelium of 4-week old mice, with medial to the right; and are stained with anti-BGA antibodies. (A) In wild-type mice, anti-BGA selectively stained the VNO axons and their terminal in the accessory olfactory bulb (AOB); no primary olfactory axons in the main olfactory bulb (MOB) expressed BGA. (B) In BGAT-Tg mice, BGA was expressed by main olfactory axons which terminated in glomeruli (arrows) in the MOB, and by VNO axons in the AOB. (C) In the main olfactory neuroepithelium of wild-type mice, neurons did not express BGA (double-headed arrow); fascicles of VNO axons within the lamina propria strongly expressed BGA (arrows). (D) In BGAT-Tg mice, cell bodies of neurons (double-headed arrow) throughout the main olfactory neuroepithelium and their axons (arrowhead) expressed BGA. VNO axons (arrow) in the lamina propria strongly expressed BGA similar to wild-type animals. Lot, lateral olfactory tract. Scale bar is 320 µm.

Fig. 2. Fasciculation of regenerating vomeronasal axons is perturbed in BGAT-Tg mice. Panels A-B are coronal sections through the vomeronasal organ; panels C-J are coronal sections through the region of the olfactory bulb or bulbectomised cavity; medial is to the right for (A-C, E) and left for (D, F-J). (A-B) Four weeks following unilateral bulbectomy, VNO neurons regenerated. KH10 immunohistochemistry confirmed that the distribution of regenerated neurons was similar in (A) wild-type and (B) BGAT-Tg mice. Sections C-J are double-labelled for OMP (red: olfactory axons) and KH10 (green: VNO axons). (C, E) Double labelling for OMP and KH10
showed that KH10 only labelled the VNO axons which terminated in the accessory olfactory bulbs (AOB) of control and BGAT-Tg mice. (D) Four weeks after surgery in wild-type mice, VNO axons partitioned from main olfactory axons on the bulbectomised side and formed large bundles of adjoining fascicles (dashed line). (F-H) In BGAT-Tg mice, VNO axons formed smaller bundles (dashed line) with numerous small fascicles interspersed with the main olfactory axons (arrows). (I-J) Higher magnification view of the plexus; (I) in control animals occasional VNO axons (arrows) were located interspersed amongst the main olfactory axons; whereas in BGAT-Tg animals (J) numerous bundles of VNO axons were interspersed amongst the fascicles of main olfactory axons. Epi, epithelium; NS, non-sensory epithelium. Scale bar is 240 µm in A-H; 720 µm in I-J.

Fig. 3. Regenerating VNO axons in BGAT-Tg mice form numerous small and dispersed fascicles. (A) The mean cross sectional area (+/- s.e.m.) of the largest bundle of VNO axons in wild-type and BGAT-Tg mice; p<0.05 for a two tail t-test. (B) The mean number (+/- s.e.m.) of VNO axon fascicles that had a diameter larger than 2.5 µm in wild-type and BGAT-Tg mice; p<0.01 for a two tail t-test.
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