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### **Inhibition of Semaphorin3A Promotes Ocular Dominance Plasticity in the Adult Rat Visual Cortex**

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(Article begins on next page)

# Molecular Neurobiology

## Inhibition of Semaphorin-3A promotes ocular dominance plasticity in the adult rat visual cortex

--Manuscript Draft--

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<b>Abstract:</b>	<p>Perineuronal nets (PNNs) are condensed structures in the extracellular matrix that mainly surround GABA-ergic parvalbumin-positive interneurons in the adult brain. Previous studies revealed a parallel between PNNs formation and the closure of the critical period. Moreover, ocular dominance plasticity is enhanced in response to PNN manipulations in adult animals. However, the mechanisms through which perineuronal nets modulate plasticity are still poorly understood. Recent work indicated that perineuronal nets may convey molecular signals by binding, and storing proteins with important roles in cellular communication. Here we report that Semaphorin 3A (Sema-3A), a chemorepulsive axon guidance cue known to bind to important perineuronal net components, is necessary to dampen ocular dominance plasticity in adult rats. First, we showed that the accumulation of Sema-3A in PNNs in the visual cortex correlates with critical period closure, following the same time course of perineuronal nets maturation. Second, the accumulation of Sema-3A in perineuronal nets was significantly reduced by rearing animals in the dark in the absence of any visual experience. Finally, we developed and characterized a tool to interfere with Sema-3A signaling by means of AAV-mediated expression of receptor bodies, soluble proteins formed by the extracellular domain of the endogenous Sema-3A receptor (neuropilin1) fused to a human IgG Fc fragment. By using this tool to antagonize Sema-3A signaling in the adult rat visual cortex, we found that the specific inhibition of Sema-3A promoted ocular dominance plasticity. Thus, Sema-3A accumulates in perineuronal nets in an experience-dependent manner and its presence in the mature visual cortex inhibit plasticity.</p>

Pisa, December 10th, 2018

Dear Editor,

We have received the review of our manuscript "Inactivation of Semaphorin-3A promotes ocular dominance plasticity in the adult rat visual cortex." We are glad that both reviewers were positive about our manuscript. For rev #4 the manuscript was already acceptable for publication, while the issues raised by rev #3 were answered by new experimental results. These novel results show that:

1. interfering with Sema3A signaling by AAV-mediated expression of soluble receptor bodies does not significantly contribute to modify the structural relationship between PNNs and Sema-3A;
2. The time course of WFA positive and Sema-3A positive PNNs progresses in parallel during postnatal development.
3. All the receptor bodies used in the study have the expected molecular weight and are effectively released in the culture medium, as shown by western blot analysis. These results, together with our data showing functional interference in growth cone collapse assays, give a better characterization of the molecular tool employed to counteract Semaphorin-3A.

We hope that now the paper is suitable for publication. A point-to-point rebuttal follows  
Best regards, Tommaso Pizzorusso

## Ref #3

### **Major points:**

*1. Title: "Inactivation" is not suitable with current strategy. "Inhibition or interference" would be more appropriate.*

A: We agree, the title was changed accordingly replacing "Inactivation" with "Inhibition".

*2. Experimental procedures:*

*Indicate number of animals used in the study as well as institutional approval of experiments.*

A: The requested info was added in the first paragraph of the Experimental Procedures

*3. Results: Reviewer requests WFA-positive PNN numbers at P14, P28, P45 and P90 to be compared with Sema-3A and WFA-double positive PNN numbers in Fig. 1E. This would clarify the correlation and time course between the formation of PNN and accumulation of Sema-3A.*

A: We have included new data providing a quantitative analysis of Sema-3A and WFA positive PNNs at different ages in the fig. 1. These new results confirm the progressive formation of perineuronal nets during postal development and show that Sema-3A aggregates onto WFA positive structures in a similarly age-dependent manner.

*- the last sentence in page 11, " suggesting that Sema-3A could inhibit plasticity in the adult rat visual cortex." remains a matter of speculation. The reduced levels of Sema-3A positive PNN may be results from reduced formation of PNNs.*

A: we agree, we have eliminated the sentence.

*- Soluble Npn receptors: Estimated molecular weights and actual protein sizes of several mutant Npn ectodomains fused with IgG Fc fragment are not indicated. Purified or concentrated recombinant proteins are better to be shown in figures although Npn1-Fc, Npn1-Y297A-Fc and Npn1-VEGF-Fc has already been reported by other groups. These results will support the main results. Otherwise, the authors' findings cannot be scientifically convincing. Biochemical experiments using the above mentioned recombinants as well as their original Npn1-T316R-Fc and Npn2-Fc proteins to examine the efficacy of soluble receptors and selective binding to Sema-3A are strongly recommended. Those could show the fundamental ability of soluble receptors. Importantly, the in vitro results may relate to the discussion of in vivo experiments.*

A: We thank the referee for prompting us to further strengthen the data supporting the use of receptor bodies for Sema-3a antagonization in vivo: we have included in Fig. 3 a western blot of the conditioned medium showing the molecular weights of the Npn1 and Npn2 receptor bodies and confirming that receptor bodies are released in the extracellular space. Moreover, we have added immunocytochemistry data showing the high specific staining for Fc in the cells transfected with the receptor bodies but not in the cells transfected with control GFP. These data add to the results of the functional growth cone collapse assay showing high selectivity of the receptor bodies in antagonizing Sema-3a effects [no effect was present when cells were treated with receptor bodies in absence of Sema-3A (Fig. 3)], and the immunohistochemistry data showing producing cells and released receptor bodies after in vivo transduction (Fig. 4). Altogether these data provide solid support for the efficacy of our approach to Sema-3a antagonization in vivo.

*Reviewer would like to know if AAV mediated Npn1-Fc and others' expression could actually interfere the Sema-3A or not and affect the numbers of Sema-3A and WFA-double positive PNNs in the visual cortex of adult rat compared with the control. This may strongly emphasize the modulation of PNNs using the current system.*

A: We thank the referee for suggesting a way to further characterize the molecular effect of Npn1-Fc on Sema-3A aggregation onto PNNs. We performed the requested experiments as follows. We treated adult rats by injections of AAVs in the visual cortex expressing Npn1-Fc and VEGF-Fc as a control. We chose VEGF-Fc because it is the most stringent control treatment that showed no significant enhancement of adult ocular dominance plasticity. Staining for WFA and Sema-3A showed that the density of Sema-3A PNNs is not significantly different between the 2 experimental groups, suggesting that the functional effect of Npn1-Fc on ODP is not mediated by a disaggregating effect toward Sema-3A on PNNs.

### ***Minor points:***

1. *Experimental procedures, there are many small mistakes probably due to the differences of authors in each paragraph.*

- GIBCO and GIBCO-Invitrogen Corp.
  - Postnatal day is normally abbreviated in capital letter (e.g. P90). Please check in page 8 (small letter) and 14 (capital letter).
  - The notations of percentile in "immunohistochemistry" are different from those in other pages.
2. Figure 4E: change "AAN-Npn1" to "AAV-Npn1".

A: These mistakes were corrected.

## **Ref# 4**

We thank the referee for appreciating our work. The manuscript was checked for grammatical errors.

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collapse assay showing high selectivity of the receptor bodies in antagonizing *Sema-3a* effects [no effect was present when cells were treated with receptor bodies in absence of *Sema-3A* (Fig. 3)], and the immunohistochemistry data showing producing cells and released receptor bodies after *in vivo* transduction (Fig. 4). Altogether these data provide solid support for the efficacy of our approach to *Sema-3a* antagonization *in vivo*.

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# Inhibition of Semaphorin-3A promotes ocular dominance plasticity in the adult rat visual cortex

**Abbreviated title:** Semaphorin-3A regulates adult OD plasticity

**Authors:** Elena Maria Boggio\*<sup>1</sup>, Erich M. Ehlert\*<sup>4</sup>, Leonardo Lupori\*<sup>3</sup>, Elizabeth B. Moloney\*<sup>4</sup>, Fred De Winter<sup>4</sup>, Craig Vander Kooij<sup>5</sup>, Laura Baroncelli<sup>1,9</sup>, Vasilis Mecollari<sup>4</sup>, Bas Blits<sup>6</sup>, James W. Fawcett<sup>7</sup>, Joost Verhaagen<sup>4,8</sup>, Tommaso Pizzorusso<sup>1,2,3</sup>

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**Acknowledgments:** The first four authors (marked with an asterisk) equally contributed to this study. This work was supported by the EU 7th Framework program (FP7) Marie Curie actions (AxRegen) 2008 - 2012 to JV.

# Abstract

Perineuronal nets (PNNs) are condensed structures in the extracellular matrix that mainly surround GABA-ergic parvalbumin-positive interneurons in the adult brain. Previous studies revealed a parallel between PNNs formation and the closure of the critical period. Moreover, ocular dominance plasticity is enhanced in response to PNN manipulations in adult animals. However, the mechanisms through which perineuronal nets modulate plasticity are still poorly understood. Recent work indicated that perineuronal nets may convey molecular signals by binding, and storing proteins with important roles in cellular communication. Here we report that Semaphorin 3A (Sema-3A), a chemorepulsive axon guidance cue known to bind to important perineuronal net components, is necessary to dampen ocular dominance plasticity in adult rats. First, we showed that the accumulation of Sema-3A in PNNs in the visual cortex correlates with critical period closure, following the same time course of perineuronal nets maturation. Second, the accumulation of Sema-3A in perineuronal nets was significantly reduced by rearing animals in the dark in the absence of any visual experience. Finally, we developed and characterized a tool to interfere with Sema-3A signaling by means of AAV-mediated expression of receptor bodies, soluble proteins formed by the extracellular domain of the endogenous Sema-3A receptor (neuropilin1) fused to a human IgG Fc fragment. By using this tool to antagonize Sema-3A signaling in the adult rat visual cortex, we found that the specific inhibition of Sema-3A promoted ocular dominance plasticity. Thus, Sema-3A accumulates in perineuronal nets in an experience-dependent manner and its presence in the mature visual cortex inhibit plasticity.

# Introduction

Perineuronal nets (PNNs) are condensed structures of extracellular matrix (ECM) that ensheath the soma, proximal axon, and dendrites of neurons in many parts of the central nervous system (CNS) [1–4]. Their maturation in the visual cortex closely matches the closure of the critical period [1,5]. Importantly, degradation of PNNs with chondroitinase ABC (chABC), has been shown to restore a juvenile-like condition in many experimental paradigms of neuroplasticity in both mice and rats, including ocular dominance (OD) plasticity and extinction of fear memories, thus establishing a causal connection between PNNs and reduced plasticity [1,6–9]. Moreover, experimental paradigms known to modulate the level of plasticity in the brain (dark rearing (DR) and environmental enrichment) have also a profound impact in PNNs regulation, reinforcing the idea that plasticity and PNNs are tightly coupled [1,10–13]. The precise molecular mechanisms that are responsible for PNNs effect on plasticity remain elusive. The great majority of the PNNs in the visual cortex of rats and mice surrounds parvalbumin (PV) positive neurons [1,14], a class of inhibitory interneurons important for OD plasticity [15–23]. A proposed model suggests that the complex structures of PNNs may act as a scaffold by binding plasticity-regulating molecules and by presenting them in high concentration to the neurons they enfold [24]. Indeed, Otx2 has been shown to bind to chondroitin sulfate chains, an abundant glycosaminoglycan (GAG) in PNNs. This binding is necessary for Otx2 internalization in PV positive, PNN-bearing, interneurons and, in turn, for critical period closure [6,25]. The PNN-binding capability of Otx2 is dependent upon a basic motif enriched in arginine-lysine (RK) doublets at its N-terminus [26]. Recent work provided evidence that Semaphorin-3A (Sema-3A), a chemorepulsive protein prominently expressed in the postnatal and adult brain, has a strong affinity for CSPGs, is concentrated in PNNs and, like Otx2, contains an arginine-lysine rich basic domain at its C-terminus [27–29]. Moreover, enzymatic or genetic disruption of PNNs integrity leads to a decline in Sema-3A-positive PNNs further confirming their binding. Within the PNN, Sema-3A interacts specifically with 4,6-disulfated form of chondroitin sulfate chains

(CS-E), a specific sulfation pattern in CS molecules [28,30]. The biological effect of Sema-3A binding to PNNs is still poorly understood, however, in dorsal root ganglia neurons, Sema-3A is known to induce growth cone collapse and neurite growth inhibition and this effect is amplified by its binding to GAGs [27]. These results suggest that Sema-3A might act as a plasticity inhibiting factor, through its accumulation in PNNs.

To test this hypothesis we interfered with Sema-3A function using secreted decoy Sema-3A receptors (“receptor bodies”) composed of the extracellular domain of the Sema-3A receptor Neuropilin1 (Npn1) fused to human IgGFc. Npn1 is a co-receptor for Sema-3A and VEGF. Therefore two mutant isoforms of Npn1, which selectively bind to Sema-3A [31] or VEGF [32], were used to discriminate between the effects of interfering with the function of Sema-3A or VEGF. Adeno-associated viral vector-mediated delivery of the receptor body scavenging Sema-3A, but not VEGF, promoted OD plasticity in the adult rat visual cortex indicating that persistent Sema-3A signaling in the visual cortex is necessary to maintain reduced levels of plasticity.

## Experimental Procedures

### Animal housing

Animals were maintained at 22°C in 12-h light-dark cycle. For dark rearing experiments, animals were reared in complete darkness from birth. Food and water were available ad libitum. All necessary efforts were made to minimize both stress and the number of animals used. A total of 62 rats were used. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Italian Ministry of Health (authorization number 1152/2016-PR).

## Preparation of Sema-3A- or Npn-conditioned medium and western blot analysis

HEK293T cells were transfected with Npn1-Fc, Npn1-VEGF-Fc, Npn1-Y297A-Fc, Npn1-T316R-Fc, Npn2-Fc or a myc-Sema-3A expression construct using the polyethyleneimine (PEI) method [27, 33] ; for 20ug DNA, 80ug of PEI was used. The culture medium (DMEM-GlutaMax, supplemented with 2% FCS and 1x PenStrep) was refreshed one day after transfection. Medium was collected 48h after transfection and processed for Western Blot analysis as follows. Samples were spun to remove cell debris and boiled with loading buffer containing 10% SDS and 10% B-mercaptoethanol and loaded onto 8% SDS-PAGE gels. A standard curve of known protein concentrations (200ng, 400ng, 800ng per well; hSema3A-Fc, R&D systems, 1250-S3) was loaded alongside the conditioned medium samples in order to determine the concentration of Sema-3A or neuropilin in the conditioned medium. Membranes were blotted with rabbit-anti-Sema-3A (1:1000, Abcam, Ab23393) or goat-anti-Fc (1:5000, Chemicon, AP113), developed using IRDye-secondary antibody (IRDye800® 1:2500, LI-COR Biosciences 925-32214) and scanned with the Odyssey Infra-red imaging system (LI-COR Biosciences). The resulting bands were analyzed for quantification purposes.

In preparation for immunocytochemistry, transfected cells were fixed with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (pH 7.4) for 15 minutes, washed with PBS and stained with the same antibodies as indicated above..

## Dorsal root ganglion explants culture and growth cone collapse assay

Glass coverslips were pre-coated overnight with 0.5 mg/ml poly-ornithine (Sigma Aldrich, P-3655). On the day of use, the glass coverslips were washed with water and subsequently coated with 40 µg/ml laminin (Invitrogen). After a 2 hour incubation at 37°C, the laminin

solution was removed, coverslips were washed briefly with DMEM/F12 medium and freshly prepared culture medium (DMEM/F12 1:1 (GIBCO) containing N2 supplement (Sigma Aldrich), 20 ng/ml NGF (Recombinant Rat beta-NGF, 556-NG-100, R&D Systems Europe Ltd.), PenStrep (GIBCO), 2 mM L-Glutamine (GIBCO)) was placed in each well before the DRG explants were added. A timed-pregnant (E15) Wistar rat was deeply anesthetized by CO<sub>2</sub>-inhalation and decapitated. The uterus was removed and placed into ice-cold Lebowitz (L15, GIBCO) medium. Under sterile conditions, embryos were removed from the uterus, and the spinal cord was dissected to reveal the dorsal root ganglia (DRGs). Individual DRGs (clean of any loose connective tissue and nerve roots) were centered on the laminin-coated glass coverslips, submerged in 500µl of culture medium. DRG explants were cultured overnight at 37°C with 5% CO<sub>2</sub> to allow for neurite outgrowth and growth cone formation.

Treatment solutions containing various quantities of GFP-, Sema-3A- and/or Npn-receptor body-conditioned medium were prepared by transfecting HEK cells with the appropriate expression plasmids and concentrating medium using an Amicon 100 kDa MWCO Ultra-15 device (Millipore). These samples were incubated on ice for 1 hour to allow for stabilization of the protein interactions and were warmed briefly (in a water bath at 37°C) before slowly applying the mixtures onto the DRGs. The volume added per well did not exceed 10% of the total volume in the well (maximum 50ul treatment mixture in a well, containing 500µl of culture medium). DRGs were incubated with conditioned medium samples for 30 minutes at 37°C. Following a 1 hour fixation in 4%PFA/10% sucrose in PBS, DRGs were washed with PBS/0.2% Triton X (PBS/Tx) and subsequently incubated with Phalloidin-TRITC (Sigma, P1951) in PBS/Tx for 3 hours at RT to visualize the actin cytoskeleton. Following a second wash in PBS/Tx, coverslips containing the DRGs were mounted onto glass slides using Mowiol 4-88 mounting medium (Sigma-Aldrich). Fluorescent images were obtained using a Zeiss Axiovert microscope, and growth cones were scored as normal (presence of lamellipodia and/or  $\geq 3$  filopodia) or collapsed (no lamellipodia,  $< 3$  filopodia or bullet shaped) based on the classification described by [34]. For each treatment group, the

percentage of growth cones was normalized to the percentage growth cones in the GFP-condition. Data represents pooled data from DRGs across 5 independent experiments.

## AAV production

Npn1 is a co-receptor for Sema-3A as well as for vascular endothelial growth factor (VEGF). We, therefore, generated AAV vectors encoding the extracellular domain of wildtype Npn1 (recognizing both Sema-3A and VEGF), and AAV vectors which harbor Npn1 mutants recognizing either Sema-3A (Npn1-Y297A-Fc; Herzog et al 2011) or VEGF (Npn1-VEGF-Fc; Gu et. al. 2002). Based on the studies of Parker and colleagues [35,36] an additional mutant (NPN1-T316R) was created in which the threonine (T) at position 316 to an arginine (R) results in a complete abolishment of VEGF-A164 binding (Npn1-T316R), and has significantly compromised Sema-3A binding in biochemical assays [37] thus potentially generating a binding-deficient receptor.

Adeno-associated viral vector transfer plasmids containing the coding sequences for Npn1-Fc, Npn1-VEGF-Fc, Npn1-Y297A-Fc, Npn1-T316R or Npn2-Fc (Moloney et. al. submitted) were used to produce adeno-associated viral vectors (AAVs). The packaging plasmids containing cap and rep genes for serotype 8 (p5E18VD2/8) and the adenovirus helper functions (pAddeltaF6) were kindly provided by Jim Wilson. A batch of AAV8 was made using the following protocol. Six 15-cm Petri dishes each containing  $1.25 \times 10^7$  HEK 293T cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS; all GIBCO) were prepared one day before transfection. The medium was refreshed 1 hour prior to transfection to Iscoves modified Eagle medium (IMEM) containing 10% FCS, 1% PS and 1% Glutamine. Transfer plasmids were co-transfected using polyethyleneimine (PEI, MV25000; Polysciences Inc., Warrington, PA, USA) in a ratio of 1:1:1 with the plasmids resulting in a total amount of 50ug of DNA per plate. The day after transfection, the medium was replaced with fresh IMEM with 10% FCS, 1% PS and 1% Glutamine. Two days later (3 days post-transfection), cells were harvested in D-phosphate buffered saline (D-PBS, GIBCO) and lysed with 3 freeze-thaw cycles. Genomic

DNA was digested by adding 10 µg/ml DNaseI (Roche Diagnostics GmbH, Mannheim, Germany) into the lysate and incubating for 1 hour at 37°C. The crude lysate was cleared by ultracentrifugation at 4000 rpm for 30 minutes. The viral vector was purified from the crude lysate using the iodixanol gradient method [38–40], diluted in D-PBS/5% sucrose and concentrated using an Amicon 100 kDa MWCO Ultra-15 device (Millipore). All AAV vectors were stored at -80°C until use. Titers (genomic copies/ml) were determined by quantitative PCR on viral DNA primers directed against the enhancer portion of the CMV promoter (Forward: CCCACTTGGCAGTACATCAA; Reverse: GGAAAGTCCCATAAGGTCATGT).

## Intracortical injections

Adult (>P90) rats were initially anesthetized with isoflurane (3%) and deeply anesthetized with an intraperitoneal injection of avertin (2,2,2-tribromoethanol, 20 µL/g). The animals were placed in a stereotaxic frame and the head was fixed with prilocaine (EMLA) covered earbars. Throughout the procedure, body temperature was monitored using a rectal probe and maintained at 37°C with a homeothermic blanket (Harvard Apparatus Ltd, Edenbridge, Kent, UK) and a constant flow (1.5 L/min) of oxygen-enriched air was delivered to the animal's head. After local disinfection of the head with povidone-iodine, the scalp was cut with a scalpel and the skin flaps were retracted. The 2 sites of injection were in correspondence to the visual cortex and their position was identified in respect to lambda (Anterior: 0 mm, Lateral: 3.8 mm); (Anterior; 2 mm, Lateral: 3.8 mm).

A 1-mm burr hole was drilled through the skull at the 2 sites with a dental drill while continuously applying cold saline over the area to prevent overheating and consequent damage to the brain. A glass micropipette with a tip diameter of approximately 30 µm was filled with 0.75 µL of an AAV solution in sterile saline. When the meninges were exposed, the injections were delivered, by piercing the underlying dura mater via the glass micropipette connected to a syringe, at a depth of approximately 0.7 mm from the brain surface at each injection site. The injection was delivered at a rate of 0.5 µL/min with a 1-min interval before

retracting the micropipette from the tissue. The scalp was then sutured with surgical suture. Animals were allowed to recover from anesthesia in a heated recovery box until fully conscious and, afterward, paracetamol (100 mg/kg) was administered in the water.

## Visually evoked potentials

Adult (>P90) rats were anesthetized with an intraperitoneal injection of urethane (0.7 ml/hg; 20% solution in saline; Sigma) and head-fixed in a stereotaxic frame. After local disinfection of the head with povidone-iodine, the scalp was cut and a portion of the skull (4x4 mm) overlying the binocular visual cortex (binocular area Oc1B), contralateral to the deprived eye, was drilled and removed, leaving the dura mater intact. The dura mater was then removed, a glass micropipette, filled with 3M NaCl (2MΩ impedance) was inserted at 3.5 to 3.8 mm lateral to lambda, and a subcutaneous ground electrode was placed in the cervical area. Throughout the procedure, body temperature was monitored using a rectal probe and maintained at 37°C with a homeothermic blanket (Harvard Apparatus Ltd, Edenbridge, Kent, UK) and a constant flow (1.5 L/min) of oxygen-enriched air was delivered to the animal. Additional doses of urethane were used to keep the anesthesia level stable throughout the experiment. Visual stimuli were computer-generated horizontal sinusoidal gratings (0.08 cyc/deg) and were presented on a monitor suitably linearized by gamma correction and positioned centrally to the vertical midline. The stimulation consisted of the abrupt contrast reversal of sinusoidal gratings (temporal frequency: 1 Hz). The receptive field of the recorded area was verified by stimulating the contralateral eye with a visual stimulus windowed to a vertical stripe in order to test the insertion of the electrode in the binocular area. Only traces responding to a windowed stimulus within a field of 20° from the vertical midline were considered.

## Immunohistochemistry

Adult rats were transcardially perfused with cold phosphate buffer (0.1M pH: 7.4) and subsequently with cold paraformaldehyde 4% (in 0.1M phosphate buffer pH: 7.4). Brains were then collected and post-fixed by incubating them overnight at 4°C in paraformaldehyde 4%. After incubation in EDTA (0.25M in PBS, 48h, 4°C) and in Sucrose (25% in PBS, 48h, 4°C), the brains were snap frozen in 2-methylbutane cooled to -40°C. The samples were embedded in Tissue-Tek O.C.T. (Sakura Finetek USA Inc.) and 40µm coronal sections were cut in a Leica cryostat and collected in Tris-buffered saline (TBS pH: 7.4).

For Sema-3A/NeuN double staining, free-floating sections were blocked by incubation in bovine serum albumin (BSA, 5%) and Triton X100 (0.2%) in TBS for 1h at room temperature (RT) and then incubated overnight at RT with Sema-3A antibody (Santa Cruz C17, 1:50, BSA 5%, Triton X100 0.2% in TBS) and for 2h RT with Cy3-conjugated anti-Goat (AbCam ab6949, 1:500, BSA 5%, Triton X100 0.2% in TBS) to complete the reaction. Afterwards, sections were counterstained with NeuN antibody (Merck Millipore MAB377, 1:500 BSA 5%, Triton X100 0.2% in TBS) and for 2h RT with Alexa Fluor 488 conjugated anti-Mouse (AbCam ab150105, 1:500, BSA 5%, Triton X100 0.2% in TBS).

For each animal, at least twelve fields from 5-12 different coronal slices were acquired with a Zeiss Apotome.2 system with a Zeiss Plan-NEOFLUAR 20X, NA:0.5 lens. The primary visual cortex was identified by comparison with reference images [41]. All the images were centered on layer 4 of the cortex. We adjusted the imaging parameters to fit the brightest slice and never changed them throughout the entire experiment to ensure the same conditions for cell counting. NeuN positive cells and Sema-3A positive nets were counted manually with the java plugin cell counter for imageJ.

## Experimental design and statistical analysis

All statistical analysis has been performed in Prism (GraphPad, 5.0). A one-way ANOVA test was used, followed by Turkey's posthoc test to compare multiple groups. Statistical significance was set at  $p \leq 0.05$ . When two groups were compared, we used an unpaired, two-tailed Student's t-test. Normality of the data and homoscedasticity were controlled using SigmaStat. All data are presented as mean  $\pm$  Standard Error of the Mean (SEM).

## Results

### Sema-3A aggregation in perineuronal nets correlates with critical period closure

Previous work demonstrated that critical period closure was correlated with the formation in the visual cortex of WFA-positive PNNs [1,14]. If Sema-3A is involved in the inhibitory action of PNNs on OD plasticity, it could be expected that the accumulation of Sema-3A in PNNs in the visual cortex correlates with critical period closure. To test this hypothesis we performed WFA and Sema-3A immunostaining at P14, P28, P45 and P90 (Fig.1). Cells were considered to be positive when a full ring of staining surrounded a cell body. The results showed that Sema-3A PNN-like staining was absent at P14 and P28, whereas Sema-3A staining begins to be present at P45, in coincidence with the closure of the rat critical period for OD plasticity. Double staining with WFA and PV confirmed that Sema-3A positive structures were WFA-positive PNNs surrounding inhibitory PV-positive interneurons (Fig. 1).

Thus, *Sema-3A* positive PNNs represent about half of the WFA positive PNNs in the adult and increase in parallel with PNN formation during development.

To further corroborate the correlation between critical period closure, PNN formation and the association of *Sema-3A* with PNN, we dark-reared (DR) rats until adulthood (P90) and we assessed *Sema-3A* staining in the visual cortex. DR is known to delay the closure of the critical period for OD as well as the formation of PNNs [1,10]. Immunohistochemical staining for *Sema-3A* showed that in DR rats there was a significant reduction in the fraction of neurons (NeuN positive cells) surrounded by *Sema-3A* (Figure 2A, B). In control animals the average fraction of *Sema-3A*-positive neurons was 3.6% (SEM=0.78%, N=3). Conversely, in dark-reared animals, this percentage significantly dropped to 1.6% (SEM=0.37%, N=7). This experiment allows to conclude that the persisting plasticity of DR rats is correlated with reduced levels of *Sema-3A* positive PNN structures. Taken together, these results indicate that the high levels of plasticity occurring during early developmental times or after DR are accompanied by a low density of *Sema-3A*-positive PNN-structures in the visual cortex.

## Soluble Npn receptors, except Npn1-VEGF-Fc, are able to inhibit *Sema-3A*-induced growth cone collapse

To test the role of *Sema-3A* in PNN of adult rats we developed a tool to interfere with *Sema-3A* binding with its receptor in the adult visual cortex. Using the soluble neuropilin1 receptor fused to a human IgG Fc fragment supplied by Prof. Roman Giger (University of Michigan, USA), we performed site-directed mutagenesis to alter specific residues that had been shown to form the basis for selective *Sema-3A* binding (Y297A mutation) (Npn1(*Sema3A*)-FC) [31]. We also adapted the “nnp12ABC” mutant, created by Gu and colleagues [32], and the “Npn1-T316R-Fc” mutant [35,36], by amplifying the ectodomain of these mutants and fusing it to a human IgG Fc fragment to create soluble receptors that bind respectively only VEGF, and potentially neither *Sema-3A* nor VEGF. These constructs were inserted into adeno-associated viral plasmids under the control of the CMV promoter. The presence of the

desired mutations was confirmed by sequencing. The Npn2-Fc construct was created by amplifying the ectodomain of the neuropilin2 gene and fusing it to a human IgG Fc fragment. Sequencing of the Npn1-Fc and the Npn2-Fc confirmed that the Npn portion is in frame with the Fc and that only one stop codon exists, positioned at the 3'end of the Fc portion. To characterize the expression of the soluble neuropilin receptors, HEK293T cells were transiently transfected with the different Npn constructs. Immunocytochemistry was performed on cells 3 days post-transfection and results indicate Npn1-Fc is expressed by the transfected cells, with a faint cloud of positive staining found outside the cells suggestive of secreted protein (Fig 3A). The Npn1-Y297A-Fc and Npn1-T316R-Fc variants showed a similar cellular expression pattern as the Npn1-Fc (data not shown). Western blot analysis on conditioned medium harvested 3 days post-transfection showed that all soluble receptors are secreted into the medium and have the expected molecular weight (Fig 3B).

The efficacy of soluble Npn receptors in counteracting Sema-3A action was tested using the classical dorsal root ganglion (DRG) growth cone collapse assay. Explanted DRG neurons from an E15 rat embryo were cultured overnight on laminin in the presence of 20ng/ml NGF. DRGs were treated for 30 minutes with GFP, Sema-3A and/or soluble Npn receptors, after which the explants were fixed and the cytoskeleton was visualized using Phalloidin-TRITC. The total number of collapsed growth cones (Fig. 3C) and intact growth cones (Fig. 3D) were manually counted and graphed to illustrate the percentage of total growth cones (Fig. 3E). Untreated and Npn-alone conditions did not differ from GFP condition. All conditions were normalized to the GFP treatment condition and represent pooled data from DRGs across 5 independent experiments.

Upon Sema-3A treatment, approximately 44% growth cones remain ( $p < 0.001$ , compared to GFP treatment). The remaining grey bars illustrate the effects of pre-incubating Sema-3A with the soluble Npn receptors (Fig. 3E). When Sema-3A and Npn1-Fc are applied in combination, 60% growth cones remain after 30 minutes of treatment (Fig. 3E,  $p < 0.01$ , compared to Sema-3A treatment alone). Npn1-Y297A-Fc, the isoform which is reported to selectively bind Sema-3A and not VEGF, is also efficient in antagonizing Sema-3A function

with 68% of growth cones remaining intact in the presence of a 1:1 molar ratio of Sema-3A and Npn1-Y297A-Fc ( $p < 0.001$ , compared to Sema-3A treatment alone). As expected, Npn1-VEGF-Fc, the isoform which should only bind VEGF, does not prevent Sema-3A-induced collapse (50% growth cones,  $p = 0.28$ , ns compared to Sema-3A treatment). Surprisingly, the Npn1-T316R-Fc variant also neutralized Sema-3A function (71% growth cones in the presence of a 1:1 molar ratio of Sema-3A and Npn1-T316R-Fc,  $p < 0.01$ ). The explanation for this may be that the T316R mutant was originally tested using a truncated Npn1 protein consisting only of the b1b2 and not the a1a2 domain [35]. It is plausible that the current Npn1-T316R-Fc variant, where the a1a2 domains are intact, “override” the effect of the T316R mutation and allow the Npn1-T316R-Fc to continue binding of Sema-3A. Since the Npn1-T316R-Fc is not a binding-neutral receptor body we excluded this mutant from further in vivo study. Lastly, Npn2-Fc, a non-conventional Sema-3A receptor [42,43], is able to significantly inhibit Sema-3A-induced growth cone collapse (56% growth cones,  $p < 0.05$ , compared to Sema-3A treatment).

## Neuropilin1-Fc promotes adult OD plasticity in the rat visual cortex

To functionally assess the role of Sema-3A in the plasticity of the adult visual cortex, we tested whether the interference with Sema-3A function, achieved by AAV8-mediated expression of Npn1-Fc in the visual cortex, was sufficient to restore OD plasticity after seven days of MD in adult (>P90) rats. We first assessed Npn1 expression in the injected rats using immunohistochemistry. As shown in Fig. 4A control sections showed low levels of expression of endogenous Npn1 in particular in layer II and V with some scattered faintly labeled cells in all other layers. The sections of the cortex injected with AAV8-Npn1-Fc shows numerous pyramidal neurons as well as other cells which express Npn1-Fc and diffuse extracellular staining of secreted Npn1-Fc covering the primary visual and extending to the neighboring cortical areas (Fig 4B,C).

After assessing *in vivo* transduction and release of NPN1-Fc, we studied whether this treatment could affect Sema-3A positive PNNs. Thus, we transduced Npn1-FC into the visual cortex of adult rats and we labeled the sections for Sema-3A and WFA. Since Npn1 is also a co-receptor for VEGF, as a control we transduced Npn1-VEGF-FC (AAV-VEGF group). This Npn-1 mutant isoform specifically interacts with VEGF [32] but not with Sema-3A, and does not interfere with Sema-3A induced growth cone collapse (Fig. 3E). Fig. 4D,E shows that Sema-3A positive PNNs were not significantly affected by Sema-3a inhibitor although a trend for a reduction was observed (AAV-Npn1 group N=5, AAV-VEGF group N=5, unpaired two-tail Student's t-test  $p=0.204$ ). This suggests that NPN1-Fc does not interrupt the association of Sema-3A with PNN but rather interferes with Sema-3A-mediated signaling.

We then assessed whether Sema-3A inhibition could affect ocular dominance plasticity in the adult rat visual cortex. Figure 4F shows the ratios of the VEP amplitude obtained by stimulating the contralateral or the ipsilateral eye (C/I ratio) in non-deprived rats or after one week of monocular deprivation. In naive non-deprived rats, the C/I ratio was around two (binocular, BIN group; C/I Ratio=  $2.05\pm 0.06$ ; N=5; ANOVA:  $p<0.0001$ ). Seven days of MD did not significantly change the C/I ratio (MD group, C/I Ratio= $2.06\pm 0.15$ ; N=4. Post-hoc:  $p>0.9$  vs Bin). Strikingly, the rats expressing Npn1-FC showed a significantly lower C/I ratio (AAV-Npn1 group; C/I Ratio=  $1.29\pm 0.08$ , N=4. Post-hoc:  $p<0.001$  vs BIN and MD) than BIN or MD rats indicating the activation of OD plasticity. As a control, rats were injected with an empty vector and monocularly deprived for seven days (AAV-empty). These rats had a C/I ratio not different from untreated BIN or MD rats, and the C/I ratios were significantly higher than observed in AAV-Npn1 rats (C/I Ratio=  $1.87\pm 0.13$ , N=5. Post-hoc:  $p>0.8$  vs BIN, MD;  $p<0.01$  vs AAV-Npn1) indicating that the injection procedure and the viral transduction did not alter plasticity levels. Since Npn1 is also a co-receptor for VEGF, we tested rats expressing Npn1-VEGF-FC (AAV-VEGF group) or Npn1-Y297A-FC (AAV-Sema-3A group), an NPN-1 mutant isoform specifically interacting with Sema-3A [31] and functionally interfering with Sema-3A induced growth cone collapse. While seven days of MD did not

significantly modify the C/I ratio in AAV-VEGF rats with respect to BIN, MD and AAV-empty groups (C/I ratio=  $1.78 \pm 0.05$ , N=4. Post-hoc:  $p > 0.5$  vs BIN, MD and AAV-empty,  $p < 0.05$  vs AAV-Npn1, AAV-Sema-3A); the receptor body specific for Sema-3A elicited a plasticity-enhancing effect (C/I ratio=  $1.19 \pm 0.11$ , N=4. Post-hoc:  $p < 0.01$  vs BIN, MD, and AAV-empty,  $p < 0.05$  vs AAV-VEGF,  $p > 0.9$  vs AAV-Npn1), mirroring the outcome of the injection of the neuropilin-1 receptor body.

We also tested another receptor body, Npn2-FC, which is based upon the isoform 2 of neuropilin. Remarkably, the injection with this construct, also allowed a shift in OD after 7 days of MD (C/I ratio=  $1.32 \pm 0.10$ , N=5; Post-hoc:  $p < 0.01$  vs BIN, MD, and AAV-empty,  $p < 0.05$  vs AAV-VEGF,  $p > 0.9$  vs AAV-Npn1 and AAV-Sema-3A), thus mimicking the effect of the previously tested isoform 1. Although the predominant class 3 semaphorin in PNN is Sema-3A, Sema-3B is also detectable in PNN (Vo et al 2013). Sema-3B is interacting with high affinity with Npn2 and the OD shift may, therefore, have been caused by functional interference with Sema-3B. Alternatively, Npn2 can bind to Sema-3A and functional interference with soluble Npn2 could also diminish Sema-3A's function (Nasarre et al. 2009; Moloney et al., submitted).

Taken together, these results demonstrate that functional interference with the signaling of Semaphorin-3A can promote OD plasticity in adult rats and also corroborates the idea/notion that extracellular chemorepulsive molecules incorporated in the PNN could actively repress cortical plasticity in the adult brain and thus participate in the mechanisms for critical period closure.

## Discussion

The role of PNNs in the plasticity of the adult visual cortex of rodents has repeatedly been investigated, however little is known about the underlying molecular mechanisms by which PNN regulate plasticity. Our data demonstrate that Sema-3A is a plasticity brake associated with PNNs in the rat adult visual cortex. Indeed, our results and previous data showed that

Sema-3A positive PNNs in the visual cortex increase in parallel with critical period closure, whereas dark rearing, ChABC treatment, or Crtl-1 deletion, interventions that preserve plasticity in visual cortical circuits, also reduce the accumulation of Sema3A-positive PNNs [29,44]. These data suggest that Sema-3A in PNNs can be actively modulated to facilitate or restrict plasticity.

To assess the functional role of Sema-3A in adult OD plasticity we developed and characterized a tool to antagonize Sema-3A action in vivo. To selectively scavenge Sema-3A, we prepared receptor bodies that were tested in the classical assay of DRG growth cone collapse, and that could be delivered to the adult visual cortex by AAV vectors intracortical injection. We found that interfering with the function of Sema-3A by expression of Npn1-Fc in the adult visual cortex promotes OD plasticity. Importantly, a plasticity-enhancing effect was also obtained following expression of a receptor body which selectively binds Sema-3A and not VEGF. No effect was observed with the empty vector or a vector carrying a mutant isoform of Npn1 able to preferentially interact with VEGF but not Sema-3A, strengthening the specificity of our results. These data mimic the enhancement in visual cortical plasticity observed after targeting PNNs by CSPG GAG digestion by means of chABC or by genetic deletion of the PNN stabilizing factors Crtl-1 and aggrecan both in rats and mice [1,6,8,44,45] suggesting that at least part of the action of PNNs on plasticity is mediated by Sema-3A.

The active role of Sema-3A in the adult visual cortex is also supported by the observation that two of its co-receptors, PlexinA1 and PlexinA4 are located on the plasma membrane of PNN-bearing PV cells constituting a microdomain closely associated with PNN bound Sema-3A [29]. The ability of PV cells to respond to Sema-3A-Plexin signaling is further supported by the abundance in PV cells of flotillin-1 [29], a lipid raft protein that is essential for Sema-3A induced growth cone turning and endocytosis [46]. It has to be underscored that all these actions of Sema-3A located within PNNs can occur together with Sema-3A independent actions of the PNN mediated by Plexin independent signaling

pathways including protein-tyrosine phosphatase-sigma, leukocyte common-related phosphatase, or Nogo receptor-1 or receptor-3 [47–49].

The inhibitory action of Sema-3A on OD plasticity could derive from a local effect on synaptic inputs onto PV cells as suggested by recent work on cerebellar and hippocampal circuits. These studies suggested that Sema-3A in PNNs can be actively modulated to facilitate or restrict plasticity [13,29,50]. Once activated, semaphorin signaling has been shown to regulate the formation and function of synaptic contacts [51,52]. Specifically, Sema-3A increases the clustering of pre- and postsynaptic proteins in cortical neurons *in vitro* [53–56]. In the context of visual cortical plasticity all these mechanisms could contribute to the experience-dependent selection of inputs onto PV cells, a cellular population that has been shown repeatedly to be involved in regulation of critical periods [15–22,53]. In particular, it has been suggested that one of the early events of the plasticity process activated by monocular deprivation in juvenile mice is pruning of excitatory inputs onto PV cells that would lead to reduced inhibition and increased activation of cortical neurons by stimulation of the open eye. This mechanism would reduce with age and its reactivation in the adult would enhance plasticity [23]. These experiments indicate that synaptic plasticity at the level of PV cells could be an upstream mechanism that could change the network properties of the visual cortical circuit [8,16] modulating plasticity levels of the entire network. It is likely that PNN bound molecules, such as Sema-3A, acts at this level to regulate plasticity levels in the adult visual cortex.

Intriguingly a plasticity-enhancing activity was detected following expression of Npn2-Fc. It is not clear whether Npn2 is able to bind Sema-3A: whereas early results showed the lack of Npn2 binding by Sema-3A [57,58] and persistence of Sema-3A mediated repulsion in the presence of soluble Npn2 receptors [59], more recent data suggests that Npn2 can bind Sema-3A and that blocking Npn2-Sema-3A interaction abolish the chemorepulsive action of Sema-3A [42]. These latter data were corroborated by the observation that soluble Npn2-Fc also inhibited Sema-3A-induced growth cone collapse *in vitro*. It is possible that redundancy may exist between the two neuropilin receptors within the Sema-3A pathway [43]. Another

possible explanation of the potentiating effect of Npn2-FC on visual cortical plasticity is that Sema-3B, that is also present in PNNs [29], or other Npn2 binding factors, such as Sema-3F, that have also been involved in plasticity [51,53]. This observation raises the possibility that many different proteins are concentrated at the PNN by the interaction with CSPG GAGs. Recent work identified a basic motif composed of 15-amino acid enriched in arginine-lysine (RK) doublets at the N-terminal of Otx2 which is responsible for Otx2 binding to PNNs [6]. This peptide motif is present in a small number proteins (Beurdeley et al., 2012; Prochiantz and Di Nardo, 2015) that could potentially be enriched in the PNN. The C-terminus of Sema-3A interacts with CSPGs [60] and the C-terminus of Sema-3A contains an RK-peptide sequence which has very high homology to the Otx2 binding site. Moreover, Sema-3A and Otx2 share the same preference for interaction with chondroitin sulfate E (CS-E) [6,28]. Thus, Otx2, semaphorins and other GAG binding proteins could be concentrated by the PNN. In this way, the PNN would become a molecular hub changing its function depending on its molecular composition. In this view, the regulation of PNN maturation and stability could represent a regulatory step to control the extracellular microenvironment surrounding PV cells and eventually plasticity of a brain circuit.

## Figure legends

**Figure 1** Sema-3A accumulates in PNN during late postnatal development. (A) Sections of the visual cortex of rats at different postnatal ages (P14, P28, P45, P90) were stained for Sema-3A, WFA (to detect PNN) and PV (to identify parvalbumin-positive inhibitory interneurons). The right-hand column shows a merge of the Sema-3A, WFA and PV staining. Sema-3A is not yet detectable in PNN at P28 but becomes increasingly visible in the cortex at P45 and P90. At P90 robust Sema-3A labeling is observed. Sema-3A-positive PNNs are associated with PV cells. Scale bar 50  $\mu$ m. (B) Quantification of the density of Sema-3A positive and WFA positive cells in the visual cortex.

**Figure 2** Dark rearing prevents Sema-3A aggregation in perineuronal nets. (A and B) Representative images of NeuN (green) and Sema-3A (red) stained slices of the visual cortex in control (N=3) and dark-reared (N=7) rats respectively. (C) In dark-reared (DR) animals, fewer of the neurons are surrounded by an aggregation of Semaphorin-3A. (\*  $p \leq 0.05$ ; Two-tailed Student's T-test)

**Figure 3** Soluble neuropilin receptors are expressed and secreted from NRP-Fc transfected cells. (A) HEK293T cells were transiently transfected with Npn-Fc expression vectors, and processed for immunocytochemistry using HRP-conjugated antibodies directed against Fc. Npn1-Fc and Npn1-VEGF-Fc are produced in the cell after transfection. (B) Medium samples were loaded onto 8% SDS-PAGE gels and blotted with anti-Fc. All soluble Npn receptors are secreted, detected by a band at 150kDa. (C-E) Functional characterization of receptor bodies in the dorsal root ganglion (DRG) growth cone collapse assay. The growth cones E15 rat embryo DRG neurons collapse in the presence of Sema-3A, the neurite tip contains no lamellipodia and  $\leq 1$  filopodium (C, arrowhead). (D) Application of a Sema-3A and Npn1-Fc mix prevents Sema-3A-induced collapse, the lamellipodia of the growth cones remains spread (open arrowhead), and  $\geq 2$  filopodia (filled arrowhead). (E) Quantification of growth cones after treatment with Sema-3A and soluble Npn receptors. All conditions were normalized to GFP (open bar). Untreated (horizontally scored bar) and Npn-alone (vertically scored bar) conditions did not differ from GFP condition. Sema-3A treatment (8.6 nM; light grey bar) results in a significant decrease in growth cones. The remaining 5 grey bars illustrate the effects of pre-incubating Sema-3A with soluble Npn receptors. From left to right: upon combined Sema-3A and Npn1-Fc treatment there is a significant increase in numbers of growth cones; Npn1-VEGF-Fc does not rescue Sema-3A-induced growth cone collapse; Npn1-Y297A-Fc and Npn1-T316R show a similar and significant ability for neutralizing Sema-3A function and lastly, Npn2-Fc treatment significantly reverses Sema-3A-induced growth cone collapse. Data were obtained from 5 independent experiments error bars were

generated using the S.E.M. and a Student's t-test was used to determine significance (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). Scale bar = 20 $\mu$ m

**Figure 4** AAV8 mediated NPN1-Fc expression in sections of the visual cortex of adult rat. (A) A section of a contralateral control hemisphere not injected with AAV8-NPN1- Fc. (B, C) Sections of the visual cortex injected with 1 $\mu$ l of AAV8-NPN1- Fc three weeks after injection at low (B) and high magnification (C). Sections were processed for immunohistochemistry for NPN1. The control sections show low levels of expression of endogenous NPN1 in particular in layer II and V with some faintly scattered cells in all other layers. The sections of the cortex injected with AAV8-NPN1- Fc shows numerous pyramidal neurons as well as other cells which express NPN1-Fc and diffuse extracellular staining of secreted NPN1-Fc. Representative sections are shown. cc is corpus callosum. Scale bar for panel B is 100  $\mu$ m, for panel C is 25  $\mu$ m. (D-E) The expression of Npn1 receptor body did not significantly alter the density of Sema-3A, WFA double positive PNNs. (F) Contra/Ipsi ratio of AAV8 injected rats after seven days of monocular deprivation. Examples of typical visual evoked potentials recordings are shown in the inset. The top and bottom rows represent respectively the contralateral and ipsilateral responses of an MD AAV-empty injected rat and of an MD AAV-Npn1 injected rat; (Scale bar: 50 $\mu$ V, 50ms). The bars represent the ratio of the contralateral and ipsilateral VEPs amplitude in different treatment groups. No injection, transfection with an empty vector, a VEGF-specific version of Npn1-FC did not evoke any OD shift after 7d MD. Conversely, the transfection with Npr1-FC or with its Sema-3A-specific version allowed for an OD shift, thus indicating the reactivation of adult OD plasticity. (\*\*\*)  $p \leq 0.001$  vs No treatment and Bin groups; One-Way ANOVA; post-hoc: Turkey's multiple comparison test) (BIN: N=5; No Treat: N=4; AAV-Npn1: N=4; AAV-Empty: N=5; AAV-:Sema-3A N=4; AAV-:VEGF N=4; AAV-Npn2: N=5)

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