Dual Sympathetic Input into Developing Salivary Glands

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Appendix

Materials and Methods:

Ex vivo organ explant cultures

Dissected submandibular and sublingual glands from mouse and human embryos were placed on permeable membranes (BD, Franklin Lakes, NJ, USA) over culture medium (DMEM - Advanced Dulbecco Modified Eagle Medium F12, Invitrogen, Waltham, MA, USA; 1% GlutaMAX, Invitrogen; 1% penicillin-streptomycin) at 37°C and 5% CO_2 for 2-4 days (Alfaqeeh and Tucker 2013). For functional experiments, embryonic murine explants at E13.5 were treated with the neurotoxin 6-Hydroxydopamine (H4381 Sigma). Two methods were used to provide a long and short exposure to the inhibitor. For short exposure 20mM 6-OHDA in 0.01% ascorbic acid was added to dissected glands for 10 minutes. The glands were then washed and cultured for 48hrs in control medium. This method has previously been used for explant cultures (Kearns et al. 2006). The negative control group, from the contralateral side of the embryo was incubated with only 0.01% ascorbic acid for 10 minutes. For long exposure, 10 and 20mM of 6-OHDA were added to the explant cultures for continuous 48h in control medium.

For rescue experiments 100 or 150ng/ml of nerve growth factor (NGF) in 1% Bovine Serum Albumin (BSA), or BSA alone, was added to cultures treated with 6-ODHA (10mM) for a prolonged period (48hrs). After 48hrs in 6-OHDA cultures were washed and cultured with BSA or NGF for a further 48 hours (96hrs total culture period). Images of each gland were taken daily to follow development, and each group consisted of a minimum of three matched pairs of glands (n=3-6 pairs per experiment). For branching analysis, the spooner ratio (ratio between the number of buds found on the last day divided by the number found at the start of culture) of treated groups was compared to contralateral control groups using a paired Student t-test (Graph Pad software).

qPCR analysis

For PCR analysis, glands were cultured in 10 or 20mM OHDA for continuous 48 hours. RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA samples were DNase-treated (DNaseI, Invitrogen), prior to cDNA synthesis that was performed using the MMLV reverse transcriptase (Invitrogen). cDNA was diluted 1:10 to make a working concentration for the qPCR reaction. Amplification of target cDNA was performed using the GoTaq qPCR Master Mix (Promega) according to manufacturer's instructions. All reactions were carried out in triplicates with the following thermocycler conditions: 1 cycle at 95ºC for 2min, 95ºC for 15sec and 60ºC for 60sec for a total of 40 cycles, and 1 cycle at 95ºC. Primer sequences are found in Table 1. Primer specificity was tested using the PrimerBlast software (www.ncbi.nlm.nih.gov). Gene expression was normalised to the housekeeping gene *Gapdh* and to corresponding experiment control groups, obtained from five different experiments for each group (n=3 glands per group).

Immunofluorescence

For whole mount immunofluorescence, dissected and fixed salivary glands were permeabilised in 0.1% Triton in buffer (PBS-Triton) for 30 minutes each wash to allow primary antibody penetration. Samples were then incubated in blocking solution for 2h at room temperature in 10% animal serum in PBS-Triton. Primary (1:200 sheep anti-Tyrosine Hydroxylase, Millipore; 1:200 mouse anti-TUBB3 clone TUJ1, R&D; 1:300 rabbit Cleaved Caspase-3; Cell Signaling) and secondary (1:500, Alexa Fluor, Invitrogen; 1:1000 DAPI, Santa Cruz Biotech) antibodies were incubated at 4°C for 2 overnights in blocking solution. Samples were washed in PBS-Triton six times for 30 minutes after each antibody, and then mounted with glycerol in PBS (1:1). For paraffin-sections of whole heads, samples were rehydrated in ethanol series, washed in buffer and incubated in EDTA pH9.0 (Dako) in a water bath at 95ºC for 20 minutes for antigen retrieval. The blocking step was performed with the same solution as whole mount for 1h at room temperature. The concentration of primary antibodies was maintained and incubated for one overnight at 4ºC. Fluorescent secondary antibodies were incubated either for 3h at room temperature in 1:300 concentration (Alexa Fluor), or for 10 minutes with an amplifying TSA kit according to manufacturer's instructions (Perkin Elmer). Results

were analysed by confocal laser-scanning microscope (TCS SP5, Leica). Confocal images were processed using Image J and Photoshop software.

Cell quantification

For TH-positive cell counting, the whole parasympathetic ganglion in the cultured glands was imaged using z stacks on the confocal (TCS SP5, Leica). 3-5 images though the ganglion for each gland were taken to allow counting of the total number of TH positive cell bodies using Fiji software. Unpaired Student t-test was performed with Graph Pad, comparing NGF- treated explant cultures at 150ng/mL with negative control groups with only BSA 1% (N=3). Significant p value was <0.05 comparing means with SEM.

Whole mount in situ hybridisation

Mouse embryonic salivary glands from E12.5 and E16.5 were dissected and fixed with 4% formaldehyde overnight at 4^{o}C, dehydrated in methanol series up to 100% and subsequently rehydrated for 30min each step. Samples were permeabilised in detergent mix for 30 minutes (1% IGEPAL from Sigma, 1% SDS from VMR, 50mM Tris-Hydrogen Chloride pH8, 1mM Ethylene Diamine Triacetic Acid pH8, 150mM Sodium Chloride, 0.5% deoxycholic acid from Sigma), followed by incubation in 50% hybridisation buffer in PBT for 10min at room temperature and then 100% hybridisation buffer for 1h at 65°C. Diluted probe *Hand2* (1-2µg/mL) was denatured at 95^{o}C for 30min and then applied to samples overnight at 65°C. Following hybridisation, the probe was removed and samples were washed four times for 30min each in Solution X (50% deonised formamide, 2 X SSC pH4.5, 1% SDS, 100mM maleic acid (Acros Organics), 150mM NaCl, 0.1% Tween-20) at 65°C. Next, samples were incubated in Solution X and MABT (1:1) for 30min at 65°C. Samples were washed in MABT twice for 30min and blocked for 1h with 2% blocking reagent (BBR) (Roche) diluted in MABT, and for a further hour in 20% lamb serum (Invitrogen) and 2% BBR diluted in MABT. Samples were incubated in a 1:1000 anti-dig alkaline phosphatase conjugated antibody (Roche) diluted in the second blocking solution at 4°C overnight. Antibody was removed by washing six times in MABT for 30min. For colour development, samples were washed in NTMT (100mM NaCl, 100mM Tris-HCl pH 9.5, 5mM Magnesium Chloride in PBS-Triton) four times for 10min and incubated in alkaline phosphatase substrate solution (Roche) in the dark

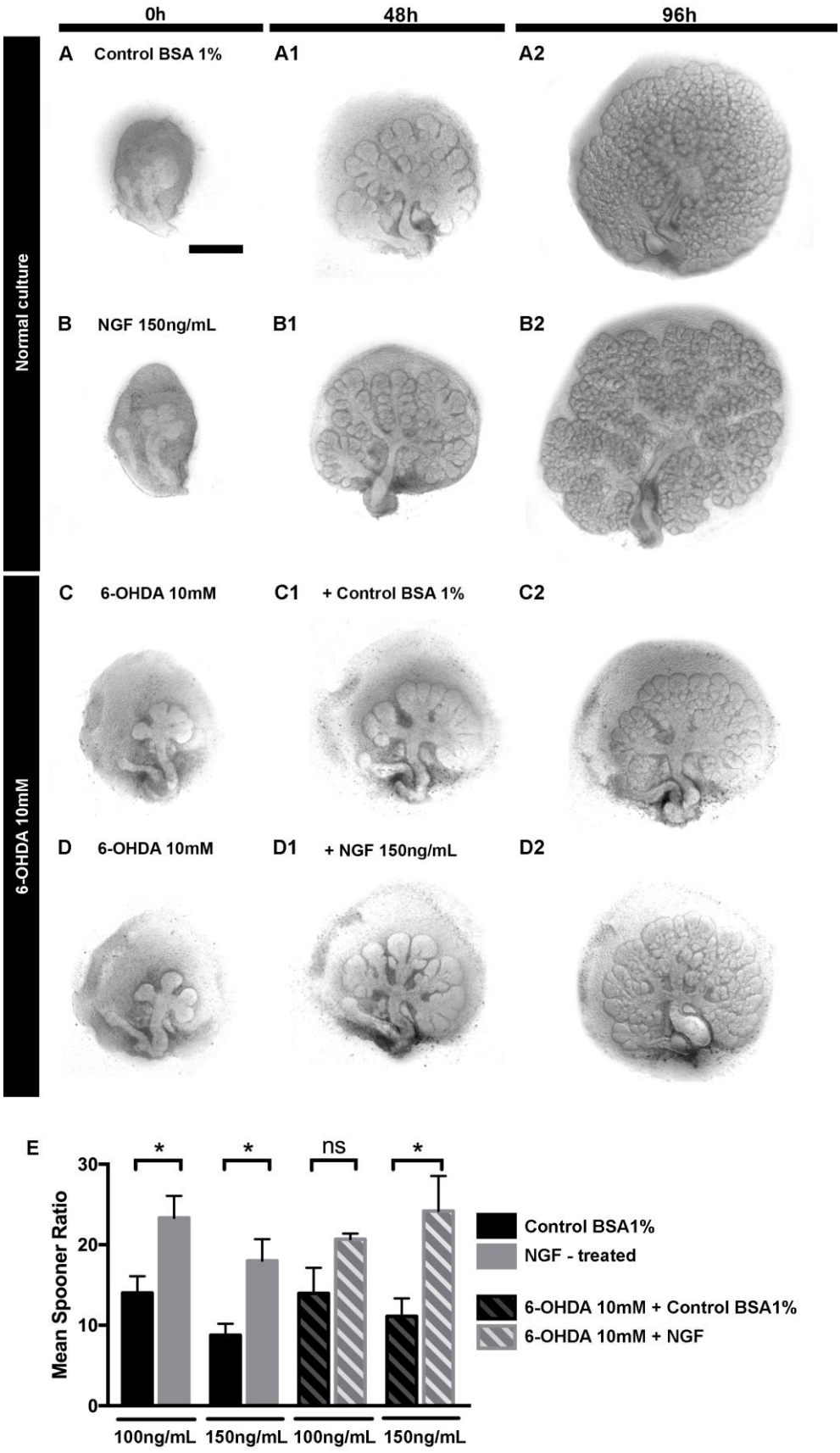
until adequate colour had developed. Samples were then rinsed in NTMT, PBS-Triton and post-fixed in 4% formaldehyde to prevent loss of signal. Dissected glands were then embedded and processed for wax sectioning.

Appendix Reference

Alfaqeeh SA, Tucker AS. 2013. The slice culture method for following development of tooth germs in explant culture. J Vis Exp.(81):e50824.

Appendix Table 1: Sequences of mouse primers used for qPCR

Gene target	Forward primer sequence	Reverse primer sequence
Gapdh	ACTCCACTCACGGCAAATTCAACGGCACAG	GGGTCTCGCTCCTGGAAGATGGTGATGGG
Tubb3	CCAGAGCCATCTAGCTACTGACACTG	AGAGCCAAGTGGACTCACATGGAG
Vacht	GAGTGGGAGATGGGCATGGTTTGG	GCAGGCAGGTACGACGCAAGAG
Vip	TCCAGTGATAGGTACTCCATCTC	CATCCATAGCACACGCAGAA



Appendix Figure 1. 6-OHDA treatment leads to defective branching can be rescued by the addition of NGF in *ex vivo* explants.

(A, B) E13.5 salivary gland cultured for 96 hours (A,B) Day 0. (A1,B1) 48 hours. (A2,B2) 96 hours. (**A**) in the presence of 1% BSA. **(B)** in the presence of 150ng/ml NGF in 1% BSA. (**C, D)** E13.5 salivary gland cultured for 48 hours with 10mM 6-OHDA. (**C, D**) Day 0. (**C1, D1**) 48 hours. Branching after treatment was delayed compared to control cultures (see **A1**). After 48 hours the 6-OHDA was washed off and NGF was added to half the cultures. (**C2**) 1% BSA added for a further 48 hours (96 total hours in culture). (**D2**) 150ng/ml NGF in 1% BSA added for a further 48 hours (96 total hours in culture). Scale bar = 500µm. (**E**) Spooner ratios to compare branching morphogenesis. Black column: Control BSA1% (see A). Grey column NGF treated (see B). Branches after 48 hours divided by number of branches on day 0. Significant difference in branching with the addition of two different concentrations of NGF (100ng/ml and 150ng/ml). (*p ≤0.05; n=3-4 pairs). Black striped column: OHDA treated followed by 1% BSA control medium after 48 hrs (see C). Grey striped column: OHDA treated followed by addition of NGF after 48 hrs (see D). Branches after 96 hours divided by number of branches at 48 hours. Significant enhanced branching with the addition NGF at 150ng/ml but not 100ng/ml (*p ≤0.05; n=3-6 pairs).