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EPIGENETIC MODIFICATION OF VOMERONASAL (V2r) PRECURSOR NEURONS BY HISTONE DEACETYLATION

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Abstract—Vomeronasal neurons undergo continuous neurogenesis throughout development and adult life. These neurons originate as stem cells in the apical zone of the lumen of the vomeronasal organ (VNO) and are described as nestin-expressing glia-like progenitor cells (Murdoch and Roskams, 2008). They then migrate horizontally along the basal zone where they differentiate into functional VNO neurons (Kaba et al., 1988). We harvested progenitor cells from the adult VNO and, after 3–6 months of *invitro* culture, these VNO neurons remained in a stable undifferentiated state expressing nestin, β-tubulin III and vomeronasal type 2 (V2r), but not vomeronasal type 1 (V1r) receptors. Application of histone-deacetylase inhibitors induced development of a neural phenotype that expressed V2r receptors, a down-regulation of nestin expression and no change in any specific genetic markers associated with glial cells. Treatment with valproic acid induced extensive changes in gene expression in the axon guidance pathway. The adult VNO is known to functionally adapt throughout life as a consequence of changes in both a mouse's physiological status and its social environment. These pluripotent cultured neurons may provide valuable insights into how changes in both physiology and environment, exert epigenetic effects on vomeronasal neurons as they undergo continuous neurogenesis and development throughout the life of a mouse.

Key words: mouse, vomeronasal organ, VNO, neurogenesis, epigenetic regulation.

Mice employ two distinct systems for the detection of olfactory cues, the main olfactory system which is used for detecting airborne odors and the vomeronasal system which is primarily used for the detection of non-volatile or volatile, protein bound pheromones. The neurons which detect pheromones in the vomeronasal organ (VNO) are unusual in that they continue to be generated throughout the life of a mouse (Graziadei and Graziadei, 1980). The vomeronasal organ has two main classes of functionally distinct pheromone detection neurons which express either V1r or V2r receptors. Some V2r receptors have become specialized to respond to peptides related to the major histocompatability complex (MHC) (Brennan and Zufall, 2006) or mouse urinary proteins (MUP's) (Hurst and Beynon, 2004; Cheetham et al., 2007), which serve as mammalian pheromones and play important roles in the regulation of behaviour and reproductive physiology (Keverne, 1999). Vomeronasal neurons do not send axonal projections to the main olfactory bulb but instead project to the accessory olfactory bulb which functions to interpret pheromonal information. There are few paradigms where neural stem cells are produced throughout the adult life of an animal and differentiate into fully functional neurons following exposure to ethologically relevant stimuli (Xia et al., 2006). There is therefore an increasing level of interest in the physiological and epigenetic signals that lead to the development and differentiation of vomeronasal stem cells into neurons.

We have successfully cultured vomeronasal progenitor neurons and have also shown that biological ligands (dilute urine or its MUP fraction) increase their proliferation and neuron survival. These biological ligands induce signalling for phosphorylation of Erk, Akt and Creb in a dose-dependent manner starting at a urine dilution of 1:500 (Xia et al., 2006). Hence, urinary pheromones which signal important information via mature VNO neurons also promote survival and proliferation of their regenerating precursor neurons. This developmental replacement and maturation of VNO neurons, and their subsequent axonal integration in the existing circuitry at the first relay in the accessory olfactory bulb (AOB), requires an understanding of the changes in gene expression that enable the precursor cells to become functional neurons. Such gene expression profiling is facilitated from the abundance of RNA that can be extracted from VNO neural cultures as they mature.

In the rat, it has been shown that co-culture of VNO neurons with AOB tissue results in the expression of olfactory marker proteins (OMP) and maturation of these neurons (Moriya-Ito et al., 2005). Further studies have identified the expression of VNO receptor genes as a consequence of co-culturing with its target tissue in the rat AOB (Muramoto et al., 2007). The fate specification of progenitor neurons has been shown to require a coordinated system of epigenetic modifications leading to chromatin remodelling (Erdmann et al., 2007), and co-ordinated changes in gene expression in order to proceed to terminal differentiation (Hsieh and Gage, 2005). Histone

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Abbreviations: AOB, accessory olfactory bulb; HDAC, histone deacetylase; IPA, ingenuity pathway analysis; MUP, mouse urinary protein; OMP, olfactory marker protein; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SB, sodium butyrate; TSA, trichostatin A; VNO, vomeronasal organ; V1r, vomeronasal type 1; V2r, vomeronasal type 2; VPA, valproic acid.

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deacetylases (HDACs) lead to the compaction of chromatin, subsequent silencing of gene transcription and control of neurogenesis (Shaked et al., 2008). It has also been shown that application of HDAC inhibitors to hippocampal neuronal progenitor cells induced a neural morphology and the expression of the neuronal markers (Hsieh and Gage, 2005). HDAC inhibitors have further been shown to promote Erk dependent cortical neuronal growth and neurogenesis (Marin-Husstege et al., 2002), a change which we have shown is brought about in the VNO neurons by the signalling pheromones in mouse urine (Xia et al., 2006).

In this study we have cultured VNO neural stem cells from adult mice. We hypothesized that deacetylation of the genome of these cells would trigger their differentiation into an adult vomeronasal neuronal phenotype. We therefore examined changes in neural morphology, the expression of specific neuronal markers and gene expression profiling following treatment with HDAC inhibitors until changes in both gene transcription and neuronal morphology stabilized.

EXPERIMENTAL PROCEDURES

Animal provision, vomeronasal cell culture and choice of HDAC treatment

In all experiments 129sv mice (females at 5 weeks of age) were used as a source of vomeronasal organs. Mice were killed by cervical dislocation. VNO's were then quickly removed and cut into small sections (less than 0.5 mm thick). The sections were immediately placed into 25 cm EasyYFlasks (Nunc, Rochester, NY, USA) containing a cell proliferation medium comprising 63% DMEM, 27% Ham's F-12, 10% fetal bovine serum, B27, 20 ng/ml EGF, 20 ng/ml bFGF and 50 U/ml penicillin/streptomycin (Invitrogen, Paisley, UK). Culture conditions were 37 °C, 5% CO_2 and 80% humidity. After the cells proliferated outwards from the edge of the VNO sections, they were treated with trypsin which caused them to detach from the plastic surface of the flask. Cells were then sub-cultured into new flasks containing fresh cell proliferation medium. Once a cell line was established, sub-culturing was carried out twice weekly to ensure the cells were in a continuously active growing state. All the cultured vomeronasal cells used in these experiments had been in continuous culture for 3–6 months after the primary cell line had been started. We initially tested the effects of three different HDAC inhibitors on vomeronasal stem cell differentiation, a working concentration of each drug: trichostatin A (TSA), 10 nM (Upstate, Co. Millipore, Watford, UK), valproic acid (VPA), 1 mM and sodium butyrate (SB), 1 μM (both Sigma, Gillingham, UK) were added to the culture medium and the cells cultured for a further 1–4 days. We found that 10 nM TSA, while inducing the differentiation of stem cells into a neuronal phenotype, lead to the death of most cell cultures within 4 days. Treatment with 1 μM SB induced a neuronal phenotype but lead to unstable levels of cell culture survival. However, the use of 1 mM valproic acid lead to stem cells differentiating into a neuronal phenotype that thrived for extended periods and the cell cultures survived extremely well. Therefore we concentrated the bulk of our work on neuronal phenotype and gene expression following VPA treatment. All mice were housed/killed in accordance with the UK Animals (Scientific Procedures) Act 1986 and EEC directive 86/609/EEC and all efforts were made to minimize the number of animals used and any potential suffering.

Immunocytochemistry

Preparation of VNO sections. Vomeronasal organs were dissected out and fixed in 4% paraformaldehyde (in phosphate buffered saline (PBS), pH 7.4) for 24 h, followed by immersion in 30% sucrose/PBS for a further 48 h. VNO's were then coronally sectioned at 30 μm thickness using a freezing sledge microtome and stored in PBS prior to immunocytochemical processing.

Preparation of cells. To encourage cells to proliferate on an easily stainable surface, sterile cover slips (12 mm diameter, 1.5 mm thickness) were placed in each well of a 24-well plate. Cells (5×10^4 cells/ml, 1 ml per well) in cell proliferation medium were then added to each well and incubated overnight, the appropriate drugs were then added to the medium the following morning and cell culture continued. Prior to immunocytochemical processing the medium was removed and 4% paraformaldehyde in PBS was added to the 24-well plate for 20 min at 4 °C. Coverslips were removed, washed and stored in PBS prior to immunocytochemical processing.

Immunocytochemistry. Sections or coverslips (covered with VNO cells) were first incubated in a blocking buffer (containing 5% goat serum, 2% bovine serum albumin and 0.1% Tween 20 in PBS, pH 7.4) at room temperature for 1 h. Primary antibodies (with dilutions) included anti-nestin (1:250, ab5968, abCam); anti-olfactory marker protein (1:250, ab62609, abCam); anti-vomeronasal receptor I (1:300, ab65644, abCam, Cambridge, UK, this antibody preferentially recognizes A1, A13 and A14); anti β-tubulin III, (1:200, T8578, Sigma); anti-glial fibrillary acidic protein (1:100, G9269, Sigma, Gillingham, UK); anti-microtubule associated protein 2 (1:1000, ab5622, Chemicon, Temecula, CA, USA). The anti-vomeronasal receptor 2 (1:200, gift from Prof. R. Tirindelli (Istituto di Fisiologia Umana, Universitá di Parma, Italy, this antibody recognizes V2R A, B and C subtypes). Sections or coverslips were incubated with the appropriate primary antibody in blocking buffer (pH 7.4) overnight at 4 °C. Sections/coverslips were washed in PBS (4×5 min each) and then incubated overnight at 4 °C with one of two fluorescent secondary antibodies either goat anti-mouse IgG H+L, Alexa fluo 568, A-11004 (red) or goat anti-rabbit IgG H+L, Alexa fluo 488, A-11008 (green) (Molecular Probes, c/o Invitrogen). These were both employed at a concentration of 1:500, with a DAPI nuclear counterstain (1 mg/1 ml) in blocking buffer. Sections/coverslips were then washed four times in PBS and mounted in aqueous Vectashield mounting medium (Vector), edges of the coverslips were sealed with nail polish. Sections were examined using confocal microscopy.

Western blotting

Extraction of proteins from cells. Cells (2×10^5/ml) were seeded in each well of a 24-well plate and cultured in cell proliferation medium overnight at 4 °C. The cells in each well were then treated with the appropriate drugs and treatment was continued for up to 4 days. At the end of treatment, the medium was removed and buffer (50 μl, 200 mM Tris, 12% SDS, 30% glycerol, 15% β-mercapoethanol and 1 mg/ml Bromophenol Blue) added to each well. Cells were thoroughly mixed with the buffer and stored at −20 °C until required.

Running acrylamide SDS gels and western blots. Mini acrylamide gels (12%) were employed and 5 μl samples were loaded in each well. Gels were run at 120 V, 0.8 mA/cm^2 for 1 h before being transferred onto a membrane using a SemiPhor semi-dry transfer unit (GE Healthcare, Chalfont St. Giles, UK).

Blotting. The membrane containing the proteins was stained with Ponceau S solution (Sigma) to examine the efficiency of protein transfer. Membranes were washed (3×10 min) in washing buffer (PBS and 0.1% Tween 20) and then blocked in 5%