Characterization of a Human Keratinocyte HaCaT Cell Line Model to Study the Regulation of CYP2S1

Alison D. McNeilly, Julie A. Woods, Sally H. Ibbotson, C. Roland Wolf, and Gillian Smith

Centre for Neuroscience, Division of Medical Sciences (A.D.M.), Biomedical Research Institute (C.R.W., G.S.), Photobiology Unit, Department of Dermatology (J.A.W., S.H.I.), and Cancer Research UK Molecular Pharmacology Unit, Biomedical Research Institute (C.R.W., G.S.), University of Dundee, Ninewells Hospital and Medical School, Dundee, United Kingdom

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ABSTRACT:

CYP2S1 is an extrahepatic cytochrome P450 (P450) that shows marked individuality in constitutive and inducible expression. CYP2S1 mRNA expression is increased in psoriasis and by treatments for psoriasis, including retinoids and UV radiation, although endogenous substrates remain poorly characterized. Because previous model systems have overexpressed modified CYP2S1 in bacteria, human HaCaT keratinocyte cells were screened for constitutive and regulatable CYP2S1 expression and CYP2S1 activity in HaCaT cells compared with a novel Chinese hamster ovary (CHO)-based cell line engineered to stably coexpress CYP2S1 and NADPH cytochrome P450 reductase. Constitutive mRNA expression for CYP2S1 and additional P450s, retinoid acid receptors (RARα, RARβ, RARγ), and retinoid X receptors (RXRα, RXRβ, and RXRγ) was assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis in HaCaT cells. Cells were then exposed to retinoids or to UV radiation (UVR), and changes in CYP2S1 mRNA abundance were further examined by qRT-PCR analysis. P450 expression in HaCaT cells was similar to human skin, with abundant CYP2S1 expression. RARα and RARγ (but not RARβ) and all RXR isoforms were also detectable. All-trans retinoic acid (atRA) induced CYP2S1 mRNA expression more potently than 9-cis RA or 13-cis RA. P450-dependent atRA metabolism was demonstrated in HaCaT cells, with a very similar metabolite profile to that produced by our CYP2S1-expressing CHO cells. CYP2S1 mRNA expression was also induced by UVR, more potently than CYP1B1, a known UVR-inducible P450. Our results demonstrate regulatable and functional CYP2S1 expression in HaCaT cells, thus identifying a human cell line model with utility for further analysis of CYP2S1 regulation and substrate specificity.

Introduction

CYP2S1 is primarily expressed in extrahepatic tissues, including lung, spleen, and the gastrointestinal tract (Rylander et al., 2001; Choudhary et al., 2006). CYP2S1 is also expressed in human skin (Smith et al., 2003b; Saarikoski et al., 2005) and shows marked interindividual variation in both constitutive expression and after exposure to UV radiation (UVR) and topical drugs, including coal tar, which contains high levels of polyaromatic hydrocarbons, and all-trans retinoic acid (atRA) (Smith et al., 2003b). Although CYP2S1 shares molecular characteristics with both CYP1 and CYP2 family enzymes, lack of detailed knowledge of substrate specificity had classified CYP2S1 as an “orphan” cytochrome P450 (P450), along with CYP2W1 and other recently discovered P450s (Wu et al., 2006). However, recent data suggest a role for CYP2S1 in the metabolism of cyclooxygenase- and lipoxygenase-derived eicosanoids (Bui et al., 2011) and in activation of the anticancer drug AQ4N (Nishida et al., 2006). CYP2S1 is also expressed in human skin, with abundant CYP2S1 expression. RARα and RARγ (but not RARβ) and all RXR isoforms were also detectable. All-trans retinoic acid (atRA) induced CYP2S1 mRNA expression more potently than 9-cis RA or 13-cis RA. P450-dependent atRA metabolism was demonstrated in HaCaT cells, with a very similar metabolite profile to that produced by our CYP2S1-expressing CHO cells. CYP2S1 mRNA expression was also induced by UVR, more potently than CYP1B1, a known UVR-inducible P450. Our results demonstrate regulatable and functional CYP2S1 expression in HaCaT cells, thus identifying a human cell line model with utility for further analysis of CYP2S1 regulation and substrate specificity.

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ABBRVIATIONS: UVR, UV radiation; atRA, all-trans retinoic acid; CPR, NADPH cytochrome P450 reductase; P450, cytochrome P450; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; RAR, retinoic acid receptor; RXR, retinoid X receptor; CHO, Chinese hamster ovary; HPLC, high-performance liquid chromatography; SSR, solar-simulated radiation.
al., 2002), and α-naphthalene, an active component of coal tar, is oxidized by CYP2S1 (Karlgren et al., 2005). However, there are conflicting reports on the role of CYP2S1 in carcino genesis metabolism. Using an Escherichia coli-based expression system, CYP2S1 was proposed to have no catalytic activity toward diverse P450 substrates including arachidonic acid, benzo[a]pyrene, benzphetamine, and, in contrast to our own data, retinoic acid (Wu et al., 2006), although functional CYP2S1 expression was not convincingly demonstrated by these authors. In contrast, increased CYP2S1 expression has been reported within bronchoalveolar cells of smokers compared with nonsmokers (Thum et al., 2006) and in activated stellate cells, myofibroblast cells associated with scar formation (Marek et al., 2007). Furthermore, CYP2S1 is differentially expressed in lesional psoriatic skin (Smith et al., 2003b) and in ovarian (Downie et al., 2005) and colorectal (Kumarakulasingham et al., 2005) tumors, suggesting a role for CYP2S1 in endogenous metabolism, which may be influenced by cell type and differentiation status.

Currently, however, a detailed characterization of CYP2S1 substrate specificity and the mechanisms regulating its expression have not been performed, in part because of lack of a reliable model system in which unmodified CYP2S1 can be expressed in a catalytically active form. The majority of previous studies have used bacterial expression systems that require N- or C-terminal modification to achieve optimum CYP2S1 expression (Karlgren et al., 2005; Bui and Hankinson, 2009). It is clear that such modifications may significantly alter CYP2S1 catalytic activity and/or substrate specificity and may additionally influence the ability of the enzyme to successfully interact with CPR.

To address these issues, we have characterized the expression and regulation of CYP2S1 in the human HaCaT keratinocyte cell line and have investigated the extent to which HaCaT cells can be used to model cutaneous P450 expression. We have also created a novel Chinese hamster ovary (CHO)-based cell line, engineered to stably overexpress CYP2S1 and CPR, using an experimental approach we have previously taken to create mammalian cell lines coexpressing various P450s, including CYP3A4, with CPR (Ding et al., 1997). Comparison of retinoic acid metabolite profiles in the HaCaT and CYP2S1-CHO cell lines has allowed us to estimate the relative contribution of CYP2S1 to P450-mediated atRA metabolism in HaCaT cells. We have also investigated the effects of UV radiation on CYP2S1 expression in HaCaT cells, to assess whether the model recapitulates UVR-mediated CYP2S1 induction in vivo, and have compared CYP2S1 regulation with CYP1B1, an additional extrahepatic P450 (cytochrome), family 26, subfamily S, polypeptide 1 (CYP1B1), which is differentially expressed in lesional psoriatic skin (Marek et al., 2007) and in colorectal (Kumarakulasingham et al., 2005) tumors, suggesting a role for CYP2S1 in endogenous metabolism, which may be influenced by cell type and differentiation status.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>TaqMan real-time PCR assay identification numbers from Applied Biosystems assay on-demand service</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Gene Symbol</td>
</tr>
<tr>
<td>P50 (cytochrome), family 2, subfamily S, polypeptide 1</td>
<td>CYP2S1</td>
</tr>
<tr>
<td>P450 (cytochrome), family 1, subfamily B, polypeptide 1</td>
<td>CYP1B1</td>
</tr>
<tr>
<td>P450 (cytochrome), family 26, subfamily A, polypeptide 1</td>
<td>CYP26A1</td>
</tr>
<tr>
<td>Retinoic acid receptor α</td>
<td>POR</td>
</tr>
<tr>
<td>Retinoic acid receptor β</td>
<td>RARβ</td>
</tr>
<tr>
<td>Retinoic acid receptor γ</td>
<td>RARγ</td>
</tr>
<tr>
<td>Retinoid X receptor α</td>
<td>RXRa</td>
</tr>
<tr>
<td>Retinoid X receptor β</td>
<td>RXRβ</td>
</tr>
<tr>
<td>Retinoid X receptor γ</td>
<td>RXRγ</td>
</tr>
</tbody>
</table>

Materials and Methods

Reagents. All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO). Reagents for real-time PCR analysis were purchased from Applied Biosystems (Foster City, CA).

Cell Culture. HaCaT cells were gifts to the Photobiology Unit from Prof. Norbert Fusenig (German Cancer Research Centre, Heidelberg, Germany) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen, Paisley, UK), 5% l-glutamate, and 5% nonessential amino acids (Sigma-Aldrich). Cells were incubated at 37°C in a humidified atmosphere of 95% CO2/5% O2.

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housekeeping gene 18s ribosomal RNA. All data presented represent fold inductions above baseline untreated cells. PCR was performed using the ABI 7700 sequence detector system as described previously (Smith et al., 2003a). Microsomal fractions (to enrich for P450 content) were prepared by repeat centrifugation. In brief, tissues were homogenized in 3 volumes of KCl (50 mM, pH 7.4) containing EDTA (1 mM) and MgCl2 (3 mM) and centrifuged at 41,000 rpm for 30 min at 4°C, microsomes were resuspended in KCl containing 0.25 M sucrose. Microsomal preparations (0.25 mg) were then preincubated with 100 nM atRA at 37°C for 5 min. The reaction was initiated by the addition of an NADPH-regenerating system at a final concentration of 1.2 mM. Reactions were carried out in a shaking water bath for 30 min and terminated by the addition of methanol (1 ml). Samples were vortexed for 2 min, and the upper organic phase was removed into a clean HPLC vial after centrifugation at 3500 rpm for 15 min at 4°C. Each assay was performed in duplicate with no cofactor and no protein controls. All experiments were performed under subdued light.

HPLC Analysis. HPLC analysis was performed as described previously (Marill et al., 2000). The reaction products were quantitatively analyzed using an HPLC system consisting of a Hewlett Packard (Palo Alto, CA) series 1100 liquid chromatograph, a reversed-phase analytical column (C18 BDS Hypersil 5 μm, 4.6 × 250 mm; Agilent Technologies, Santa Clara, CA), a Hewlett Packard series 1100 absorbance detector set at 350 nm, and a Hewlett Packard ChemStation integrator. Elutions were performed using a gradient with solvent A (1% ammonium acetate in water) and solvent B (methanol) as follows: after equilibration at 65% B and 35% A, B was increased to 100% in 35 min and re-equilibrated to initial conditions in 5 min at a flow rate of 1 ml/min.

Irradiation of HaCaT Cells. HaCaT cells were seeded in six-well plates at a density of 6 × 10⁴ cells per well and left to adhere for 24 h. The following day, the medium was changed to phenol red-free Hanks’ balanced salt solution (Invitrogen), and the cells were exposed to specific UV sources using the dose range(s) equivalent to those used previously (Villard et al., 2002) and detailed in Table 2. Cells were irradiated with UVA [Cosmedico 1550 100W, glass filtered, irradiance was measured using a psoralen-UVA meter (Waldmann, Villingen-Schwenningen, Germany; model 585100) through the filter and cell culture plate lids], UVB [Philips (Guildford, Surrey, UK) TL12 or TL01, irradiance was measured using an International light meter (International Light Technologies, Leatherhead, Surrey, UK), model IL 1400A, with filter and quartz diffuser appropriate to the source], or solar simulation (150 W xenon arc lamp with copper sulfate solution as an infrared filter and a second WG305 filter for whole spectrum; power supplied by model 406/01; Applied Photophysics Ltd., Surrey, UK). UV meters were calibrated to each source in our United Kingdom Accreditation Service-accredited optical physics laboratory using a double-grating spectroradiometer (Bentham Instruments Ltd., Reading, UK) (Traynor et al., 2005). Control cells were wrapped in tin foil and sham irradiated for equivalent time periods to those required to deliver the highest dose chosen for each light source. Additional control cells were not sham irradiated but left in the dark for the duration of the irradiation treatment.

After UVR exposure, cells were washed in PBS and cultured in normal growth medium containing serum for 48 h. The cells were then harvested, and transcript abundance was assessed by quantitative real-time PCR analysis as described above. All experiments were performed in triplicate. Values are corrected for control data. Significant cell death did not occur at these doses as assessed by neutral red uptake assays as described previously (Traynor et al., 2005).

### Table 2

Light sources and dose ranges used in experiments

<table>
<thead>
<tr>
<th>Light Source</th>
<th>Spectrum</th>
<th>Irradiance</th>
<th>Dose Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solar simulation</td>
<td>290–700</td>
<td>1350</td>
<td>0.5, 1.0 J/cm²</td>
</tr>
<tr>
<td>UVA</td>
<td>320–420</td>
<td>2.7</td>
<td>0, 1.5, 3, 5, 7.5 J/cm²</td>
</tr>
<tr>
<td>UVB</td>
<td>290–320</td>
<td>0.92</td>
<td>10, 20, 40 mJ/cm²</td>
</tr>
<tr>
<td>Narrowband UVB (TL-01)</td>
<td>311–313</td>
<td>1.0</td>
<td>1.5, 3, 5, 7 J/cm²</td>
</tr>
</tbody>
</table>

**Data Presentation and Statistical Analysis.** Values are expressed as relative abundance (arbitrary units), where CYP2S1 expression has been normalized to the expression of 18S ribosomal RNA; all data represent the mean ± S.E.M. of triplicate measurements in three different passages of cells. “Fold induction” represents the differences in CYP2S1 expression in control and treated cells. Only fold change >2 was included in the analysis. Where two groups were studied, data were analyzed by Student’s t tests (parametric data) or the U test (nonparametric data). Where three or more groups were studied, data were analyzed by one-way analysis of variance followed by post hoc least-squared difference tests. Significance was arbitrarily defined as *p* < 0.05. All analyses were performed using Statistica (version 9; StatSoft Ltd., Tulsa, OK).
Atterbury, Milton Keynes, UK) or GraphPad Prism (version 4; GraphPad Software Inc., San Diego, CA).

Results

P450 Expression in HaCaT Cells. Quantitative RT-PCR analysis was used to investigate which of the major “drug-metabolizing” P450s was expressed in HaCaT cells. Transcripts for 9 of the 12 P450 genes assessed and for CPR were detected (Fig. 1A), whereas CYP2C8, CYP2A6, and CYP2D6 were below the limit of detection (i.e., no amplification after 40 cycles of PCR) and were, therefore, not analyzed further. CYP2S1 and CYP26A1, the main P450 enzymes involved in retinoic acid metabolism along with CYP1B1 and CYP2E1, were the most abundant P450s expressed in HaCaT cells, in keeping with previous findings in human skin (Fig. 1C) (Smith et al., 2003b). Although there were quantitative differences in P450 expression in HaCaT cells and in human skin, it was of particular interest to note that CYP2S1 expression was uniquely enriched in HaCaT cells compared with the expression pattern in human skin and that HaCaT cells also expressed CPR at a level equivalent to that seen in human skin.

Retinoid Receptor Expression in HaCaT Cells. Analysis of RAR and RXR isoforms by quantitative RT-PCR confirmed the presence of RARα/H9251, RARβ/H9253, RXRα/H9251, RXRβ/H9252, and RXRγ/H9253 in HaCaT cells. In agreement with previous reports of gene expression in human skin (Feng et al., 2006), RXRα/H9251 was the most abundant retinoid receptor expressed in HaCaT cells, whereas RARβ/H9252 was undetectable (Fig. 2).

Induction of CYP2S1 mRNA by Retinoids. Treatment of HaCaT cells with atRA increased transcript abundance of both CYP2S1 and CYP1B1 (previously shown to be induced by atRA) (Kawai et al., 2003) (p < 0.05) above baseline untreated cells, with highest gene expression at 25 μM atRA (CYP2S1, p = 0.035 versus untreated cells; CYP1B1, p = 0.041) (Fig. 3A). In contrast, the 9- and 13-cis stereo-isomers of retinoic acid did not significantly induce CYP2S1 or CYP1B1 (Fig. 3, B and C).

Metabolism of atRA. CYP2S1 previously has been shown to metabolize atRA, using a relatively inefficient E. coli-based CYP2S1 expression system (Smith et al., 2003b). Therefore, the ability of CYP2S1 to metabolize atRA in HaCaT cells was assessed by HPLC analysis after incubation of HaCaT cells with atRA and an NADPH-regenerating system. Figure 4A represents a typical HPLC chromatogram demonstrating the appearance of various retinoic acid metabolites after a 45-min incubation period. Several metabolites were detected, which, because of a lack of commercially available standards, are labeled M1, M2, M3, and M4. By comparing retention times with our E. coli-based data and data from other investigators (Marill et al., 2000), we propose that M1 and M2 represent 4-hydroxy-atRA and 18-hydroxy-atRA, respectively.

Because HaCaT cells express a variety of P450s, it is possible that the atRA metabolites detected could be formed by a combination of P450s, although CYP26, CYP2D6, and CYP2S1 previously have been most consistently associated with atRA metabolism. To address the relative contribution of CYP2S1 to atRA metabolism, we compared atRA metabolism in HaCaT cells and in a novel mammalian cell line coexpressing CYP2S1 and CPR. CYP2S1 expression in CHO/CYP2S1/CPR cells was confirmed by quantitative RT-PCR (Fig. 4B) and Western blot analysis (Fig. 4C) and by the presence of correctly folded catalytically active CYP2S1 by reduced carbon monoxide difference spectroscopy (Fig. 4D). CHO/CYP2S1/CPR cells also efficiently metabolized atRA and generated a metabolite profile very similar to that in HaCaT cells.
similar to that obtained from HaCaT cells, where both CYP2S1 and CYP26A1 are expressed, supporting a significant role for CYP2S1 in atRA metabolism (Fig. 4E).

**Induction of CYP2S1 mRNA by UVR.** CYP1B1 previously has been shown to be induced by UVR (Katiyar et al., 2000), and we have previously reported induction of CYP2S1 mRNA expression in human skin in vivo (Smith et al., 2003b). To determine whether HaCaT cells also supported UVR induction of CYP2S1 expression, HaCaT cells were exposed to a variety of UVR simulators, and transcript abundance of CYP2S1 and CYP1B1 (positive control) were assessed. Exposure of HaCaT cells to solar-simulated radiation (SSR; 1 J/cm²) resulted in a small but significant increase in transcript abundance of CYP2S1 \( (p = 0.042 \text{ versus control}) \) and CYP1B1 \( (p = 0.032 \text{ versus control}) \). Induction of gene expression was dose and threshold dependent, with lower levels of induction of CYP2S1 and CYP1B1 at 0.5 J/cm² compared with 1 J/cm² (Fig. 5A). Likewise, exposure of HaCaT cells to broadband UVB radiation increased transcript abundance of CYP2S1 and CYP1B1 in a dose-dependent manner (CYP2S1, \( p = 0.031 \); CYP1B1, \( p = 0.046 \) versus control; Fig. 5B). Treatment with a narrowband UVB source (TL-01; 311–313 nm) used specifically for the treatment of psoriasis had a modest effect on CYP2S1 only at the highest dose \( (400 \text{ mJ/cm²}) \) used in this study (CYP2S1, \( p = 0.039 \) versus control; Fig. 5C). In contrast to UVB, transcript abundance of CYP2S1 and CYP1B1 were unaltered after exposure to UVA (Fig. 5D), suggesting that the increase in expression observed using SSR is likely to be UVB mediated.

**Discussion**

The endogenous function and contribution of CYP2S1 to xenobiotic metabolism has not yet been fully described, although recent studies have suggested a role in lipid peroxidation (Bui et al., 2011). Original dot blot analysis revealed high CYP2S1 expression in the lung, trachea, small intestine, spleen, and gut (Rylander et al., 2001). This profile has been extended to include the skin where CYP2S1 is one of the most abundant P450s expressed and CYP2S1 expression is induced by topical drugs including atRA, by UVR, and in lesional psoriasis (Smith et al., 2003b). However, the lack of suitable model systems in which to investigate CYP2S1 substrate specificity and the mechanisms regulating CYP2S1 expression has held back progress in this field.

The majority of previous studies investigating CYP2S1 have used bacterial systems in which high levels of enzyme can be generated. However, successful bacterial expression requires significant N-terminal modification of CYP2S1, and previous studies have reported difficulties in producing sufficient quantities of correctly folded heme-bound enzyme (Smith et al., 2003b; Wang et al., 2005; Bui and...
and RAR reported in human skin, with RXR expressing the same complement of RAR and RXR isoforms as that of the most abundant P450 isoform expressed. Furthermore, HaCaT cells express functional transcription factor complexes including the Ah receptor, responsible for mediating the effects of dioxin and other xenobiotics, including cigarette smoke (Wang et al., 2005) or known P450 substrates including polycyclic aromatic hydrocarbons (Smith et al., 2003b).

This study has shown that HaCaT cells exhibit a remarkably similar P450 expression profile to that of human skin, with CYP2S1 being the most abundant P450 isoform expressed. Furthermore, HaCaT cells express the same complement of RAR and RXR isoforms as that reported in human skin, with RXRα being the most abundant isoform and RXRβ undetectable (Redfern and Todd, 1992).

The majority of known CYP2 family members are localized to the outermost differentiated layers of the skin where they are thought to act as a protective barrier to carcinogens and other xenobiotes on P450 induction (for review, see Hankinson, 1995).

UVB irradiation previously has been shown to induce CYP1A1 and CYP1B1 mRNA and protein expression in human epidermis (Goerz et al., 1996; Katiyar et al., 2000; Villard et al., 2002). Likewise, Villard et al. (2002) demonstrated that broadband UVB exposure (20 mJ/cm²) increased human CYP1B1 mRNA in primary keratinocytes and HaCaT cells. The mechanisms through which UV radiation induces P450 gene expression are poorly understood. It has been proposed that UV-induced photoproducts act as AhR agonists, suggesting that induction may be mediated via the AhR pathway (Wei et al., 1999). Our results confirm these CYP1B1 effects but report a more significant induction of CYP2S1 mRNA, predominantly by broadband UVB sources, in HaCaT cells. Because UVB is known to induce CYP1A1 mRNA expression via an AhR-dependent mechanism, it is not unreasonable to suggest that the effect of UV on CYP2S1 expression occurs via a similar mechanism. This observation is consistent with our previous report that CYP2S1 expression is also induced in human skin after topical treatment with coal tar, an abundant source of polycyclic aromatic hydrocarbons (Smith et al., 2003b).

Recent evidence suggests that CYP2S1 catalytic activity is dependent on electron donation from cumene hydroperoxide, hydrogen peroxide (Bui and Hankinson, 2009), or fatty acid peroxidases (Bui et al., 2009) in an NADPH-independent mechanism, i.e., that it is independent of coupling to CPR. In contrast, however, our data clearly demonstrate that a CYP2S1/CPR coexpression system is functional. Surges in H₂O₂ concentrations during periods of oxidative stress induced via UVR irradiation or an inflammatory response such as is evidence in psoriasis may increase CYP2S1 activity and may, therefore, rationalize the changes in CYP2S1 expression that we have reported in psoriasis. Therefore, we highlight the need to perform additional studies investigating CYP2S1 expression in HaCaT cells after treatment with various electron donors.

In summary, we have demonstrated that CYP2S1 is highly expressed in HaCaT human keratinocyte cells and is induced by atRA and broadband UVB irradiation in a similar fashion to that reported in human skin in vivo. Therefore, this cell line may provide a readily accessible and relevant model in which to further investigate CYP2S1 substrate specificity and the molecular mechanisms responsible for regulating CYP2S1 expression.

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Authorship Contributions

Participated in research design: McNeilly, Ibbotson, Wolf, and Smith.

Conducted experiments: McNeilly.

Contributed new reagents or analytic tools: Woods.

Performed data analysis: McNeilly and Smith.

Wrote or contributed to the writing of the manuscript: McNeilly, Woods, Ibbotson, Wolf, and Smith.
References


Address correspondence to: Dr. Alison D. McNieIlly, Centre for Neurosci, Division of Medical Sciences, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK. E-mail: a.d.mcnieil@dundee.ac.uk