University of Bath

Chronic administration of 13-*cis*-retinoic acid does not alter the number of serotoninergic neurons in the mouse raphe nuclei.

Cheney J.G. Drew[1†], Kally C. O'Reilly[2†], Michelle A. Lane[3] and Sarah J Bailey[1*].

1 Department of Pharmacy and Pharmacology, University of Bath, Bath, UK.

2 Institute of Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas, USA.

3 Department of Family and Consumer Sciences, Nutrition and Foods Program, Texas State University- San Marcos, San Marcos, Texas, USA.

* Corresponding author: Dr Sarah Bailey, Department of Pharmacy and Pharmacology, University of Bath, Bath, BA2 7AY, UK. Tel +44 1225 386842; Fax +44 1225 386114

† These authors contributed equally to this work.

Keywords

Retinoids, dorsal raphe nucleus, depression, stereology, 5-hydroxytryptamine, tryptophan hydroxylase

Abbreviations

5-HT, 5-hydroxytryptamine; 5-HTT, 5-hydroxytryptamine reuptake transporter; 13-*cis*-RA, 13-*cis*-retinoic acid; DRN, dorsal raphe nucleus; MRN, median raphe nucleus; PBS, phosphate buffered saline; SSRIs, selective serotonin reuptake inhibitors; TPH2, tryptophan hydroxylase 2;

ABSTRACT

The synthetic retinoid 13-*cis*-retinoic acid (13-*cis*-RA), prescribed for the treatment of severe nodular acne, has been linked to an increased incidence of depression. Chronic treatment studies in rodents have shown that 13-*cis*-RA induces an increase in depression-related behaviours and a functional uncoupling of the hippocampus and dorsal raphe nucleus (DRN). Changes in the number of serotoninergic neurons in the DRN have been reported in depressed human patients. Given that retinoids have apoptotic effects, we hypothesised that a decrease in the number of serotoninergic neurons in the DRN or median raphe nucleus (MRN) would lead to decreased serotoninergic tone and in turn to the behavioural changes seen with 13-*cis*-RA administration. Here, we used immunolabelling and unbiased stereological methods to estimate the number of serotonin (5-hydroxytryptamine, 5-HT) neurons in the MRN and DRN of vehicle control and 13-*cis*-RA-treated adult mice. In the MRN, the number of 5-HT immunolabelled cells was 1815 ± 194 in control, compared with 1954 ±111 in 13–*cis*-RA treated tissues. The number of 5-HT immunolabelled cells was much higher in the DRN, with 7148 ± 377 cells in the control, compared with 7578 ± 424 in the 13-*cis*-RA treated group. Further analysis of the DRN revealed that there were no changes in the number of 5-HT neurons within distinct subregions of the DRN. Similarly, changes in the density of serotoninergic neurons or in the volume of the MRN or DRN were not observed in 13-*cis*-RA treated animals. These data show that apoptotic actions of 13-*cis*-RA do not occur *in vivo* at drug concentrations that induce changes in depression-related behaviour and functional uncoupling of the DRN and hippocampus. The potential pro-depressant behavioural and molecular effects associated with chronic administration of 13-*cis*-RA may result from changes in serotoninergic activity rather than changes in the number of serotoninergic neurons.

INTRODUCTION

Vitamin A and related retinoids are increasingly recognized to play an important role in adult brain function (Lane and Bailey, 2005). Studies have shown that retinoid signalling influences learning and memory (Etchamendy et al., 2001, Cocco et al., 2002), sleep (Maret et al., 2005, Kitaoka et al., 2007), locomotor activity (Krezel et al., 1998) and mood (O'Donnell, 2004, Bremner and McCaffery, 2008, O'Reilly et al., 2008). The synthetic retinoid 13-*cis*-retinoic acid (13-*cis*-RA, isotretinoin, Accutane) is prescribed for the treatment of severe nodular acne (Zouboulis and Piquero-Martin, 2003). Controversially, the use of 13-*cis*-RA has been reported to increase the incidence of depressive illness in approximately 1-10% of patients receiving the drug (Hull and D'Arcy, 2005, Bremner and McCaffery, 2008). In animal studies chronic administration of 1 mg/kg 13-*cis*-RA has been shown to increase depression-related behaviours in both mice and rats (O'Reilly et al., 2006, Trent et al., 2009) but not in all studies of the adult rat (Ferguson et al., 2005, 2007). However, the mechanism by which 13-*cis*-RA treatment can influence depression-related behavioural changes remains poorly understood.

We have recently shown that the administration of 13-*cis*-RA in mice for six weeks leads to metabolic changes in the inferior rostral linear nucleus of the raphe and a functional uncoupling between the raphe nuclei and the hippocampus (O'Reilly et al., 2009). One explanation for this is that 13-*cis*-RA treatment adversely affects serotoninergic input to forebrain regions. Retinoid signalling plays a crucial role in neuronal proliferation, differentiation and apoptosis during development of the nervous system (Maden, 2007). Similar actions of retinoid signalling have been proposed to occur in the mature brain. In the hippocampus of young adult mice treated with 13-*cis*-RA, adult neurogenesis was suppressed (Crandall et al., 2004). In cultured hippocampal neurons, high concentrations of 13-*cis*-RA reduced the number of neurons and the degree of dendritic branching (Liu et al., 2008). A similar effect on the morphology of serotoninergic neurons was observed in slice cultures treated with high concentrations of 13-*cis*-

RA (Ishikawa et al., 2008). Taken together these data suggest that long-term treatment with 13-*cis*-RA may lead to reduced serotoninergic input to the brain regions which mediate depression-related behaviours.

A large body of evidence supports the involvement of serotonin (5-hydroxytryptamine, 5-HT) in the pathogenesis of depression (Mann, 1999, Neumeister et al., 2004). Acute tryptophan depletion transiently reduces 5-HT synthesis and induces relapse in depressed patients (Delgado et al., 1991, Bell et al., 2001). Activation of somatodendritic G_i/G_o-protein coupled $5HT_{1A}$ autoreceptors inhibits electrical activity in serotoninergic neurons, 5-HT synthesis and 5-HT release from nerve endings (Blier and de Montigny, 1987, Sharp et al., 1989). Both increased and decreased expression of the 5-HT_{1A} receptor have been reported in depressed and suicidal patients (Neumeister et al., 2004). In addition, polymorphisms in the promoter of the human 5-HT_{1A} receptor have been linked with depression in some (Lemonde et al., 2003) but not all (Arias et al., 2002) patients. The 5-HT transporter (5-HTT) acts as a critical regulator of serotonin signalling via reuptake of 5-HT from the synaptic space (Blakely et al., 1991). Abnormalities in the expression or function of the 5-HTT may also contribute to depression. One 5-HTT polymorphism is associated with lower 5-HTT expression and anxiety related traits (Lesch et al., 1996) and with increased rates of depression and suicidality (Caspi et al., 2003). Additionally, the antidepressant selective serotonin reuptake inhibitors (SSRIs) act by blockade of 5-HTT to enhance serotoninergic neurotransmission (Blier and de Montigny, 1994). Previous work by our group, *in vitro*, has shown that 13-*cis*-RA administration leads to an increase in protein levels of the 5-HT_{1A} receptor and the 5-HTT, as well as increasing intracellular 5-HT content (O'Reilly et al., 2007). Such changes are likely to influence the activity of serotoninergic neurons and hence serotoninergic input to the forebrain.

The serotonin system in the brain originates in the raphe nuclei and projects throughout the brain to innervate forebrain regions including the hippocampus and frontal cortex (Jacobs

and Azmitia, 1992, Barnes and Sharp, 1999, Lechin et al., 2006). The median raphe nucleus (MRN) innervates the dorsal hippocampus, medial septum, nucleus accumbens core and hypothalamus. The dorsal raphe nucleus (DRN) innervates the fronto-parietal cortex, amygdala, lateral septum, nucleus accumbens shell, ventral hippocampus and hypothalamus. The DRN thus provides the main source of serotoninergic input to the cortico-limbic structures that are associated with mood disorders (Michelsen et al., 2007, Drevets et al., 2008). A decrease in the overall numbers of serotoninergic neurons in the DRN of depressed patients has been reported (Baumann et al., 2002) although others have reported increased numbers of serotoninergic neurons in suicide patients (Underwood et al., 1999). We hypothesised that chronic treatment with 13-*cis*-RA may cause a decrease in the number of serotoninergic cells emanating from the DRN, thus reducing serotoninergic input to the cortico-limbic structures implicated in mediating depression-related behaviours. In this study we have used an unbiased stereological procedure to estimate the volume and number of serotoninergic neurons in the MRN and DRN of young adult mice with or without 13-*cis*-RA chronic treatment. To the best of our knowledge this is the first report of a detailed stereological analysis of the adult mouse raphe nuclei.

EXPERIMENTAL PROCEDURES

Animals

Experiments were conducted with DBA/2J young adult male mice obtained from Jackson Laboratories (Bar Harbor, ME) and Charles River Ltd (Margate, UK). Mice were received at postnatal day 21 and treatment began at postnatal day 28 and continued for six weeks. All mice were housed in groups of four per cage with *ad libitum* access to food and water and maintained on a 12:12 h light dark cycle. All of the procedures performed were approved by the University of Texas IACUC, protocol number 04100403, according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and in the UK performed under a project licence held under the Animals (Scientific Procedures) Act 1986 and in accordance with the UK Home Office guidelines.

Drug Treatment

Animals were randomly assigned to control or drug treatment groups and allowed to acclimatize to their environment for one week before commencing treatment. Drug treatment was essentially as described previously (O'Reilly et al., 2006). All mice received an intra-peritoneal injection of either vehicle (0.9% w/v sodium chloride/ dimethyl sulphoxide (DMSO) at a ratio of 1:1 v/v) or 1mg/kg 13-*cis*-RA, dissolved in vehicle, daily for six weeks. This treatment regime has been shown to induce an increase in depression-related behaviours in young adult mice (O'Reilly et al., 2006). This dose of 13-*cis*-RA is in the range of doses widely used to treat acne in patients (0.5 to 2 mg/kg/day) and achieves plasma levels similar to those seen in patients (Kerr et al., 1982, O'Reilly et al., 2006).

Tissue preparation

At the end of treatment, mice were anaesthetized and perfused transcardially with 4% paraformaldehyde. The brains were immediately removed intact and postfixed in 4% paraformaldehyde overnight followed by storage in PBS with 0.1% azide at 4°C. Brains were cut in the caudal-rostral direction across the region containing both the MRN and DRN (-5.20 to -4.04 mm from Bregma, (Paxinos and Franklin, 2001) using a vibrotome to achieve 40 μm coronal sections that were stored in PBS with 0.1% azide at 4°C until immunohistochemistry was performed.

5-HT Immunohistochemistry

For immunochemistry every third section across the region containing both the MRN and DRN was processed. All washes were done in PBS at room temperature unless otherwise stated. Sections were washed and then blocked for endogenous peroxides (3:1 methanol: 3% hydrogen peroxide) followed by incubation in SuperBlock (Pierce, Rockford, IL) for 15 min. Subsequently, sections were incubated in blocking solution (BS) consisting of PBS containing 10% SuperBlock, 10% normal goat serum , 2% bovine serum albumin, and 0.2% Triton X-100 with a 1:10,000 dilution of the rabbit polyclonal primary anti-5-HT antibody (Sigma, Saint Louis, MO) for 24 h. The sections were then washed and incubated for 1 h in biotinylated goat anti-rabbit secondary antibody (Sigma, Saint Louis, MO) at a 1:600 dilution in BS. After washing, immunolabelling was detected using avidin-conjugated horseradish peroxidase with diaminobenzidine as substrate using the Vectastain ABC kit (Vector Labs, Burlingame, CA) according to the manufacturer's protocols. Sections were mounted onto slides and allowed to air

dry prior to Nissl counterstaining and dehydration through alcohols (75% to 100%), cleared in xylene and coverslipped with Permount mounting solution (Fisher Scientific).

Stereological analysis of 5-HT immunolabelled neurons

Using Nissl counterstained sections the MRN and DRN were outlined at a low magnification (4X objective) with the aid of a mouse atlas (Paxinos and Franklin, 2001). To demark the MRN, the anterior or ventral tegmental nucleus was used to determine the most dorsal region of the MRN, while the paramedian raphe nuclei and the tectospinal tract were used to determine the edges along the dorsal-ventral axis. The most ventral region of the MRN was identified by locating the pericentral reticulotegmental nucleus of the pons. The slices of the MRN used for the stereological analysis started at approximately -4.04 mm from Bregma and continued posterior to -4.96 mm from Bregma. The landmark used to help identify the most ventral region of the DRN was the superior cerebellar peduncle and the caudal linear nucleus of the raphe. The aqueduct was used as a landmark to determine the dorsal boundary of the DRN and sections used for stereological analysis were identified at -4.04 mm from Bregma and continued until -5.02 mm from Bregma.

The stereological analysis performed was similar to that described in (Chakraborty et al., 2003). The stereological analyses were performed using a computer-assisted morphometry system consisting of an Olympus BX61 photomicroscope (Center Valley, PA) and StereoInvestigator morphometry and stereology software (MicroBrightField, Inc., Colchester, VT). Stereological microscope analyses were performed at high magnification (100X oil objective, N.A. 1.35) and approximately nine slices were analyzed per subject for the MRN and 10 slices were analyzed per subject for the DRN. The observer was blinded as to which treatment group the subject belonged to during the stereological analysis.

The StereoInvestigator software placed dissector frames using a systematic random design within each contour outlining each region on a 90 x 90-μm^2 grid for the MRN and a 90 x 130-μm^2 grid for the dorsal raphe nuclei. 5HT labeled cells were counted within 45 x 45 μm optical dissector frames on the x-/y- axis and the final post-processing thickness of each section was measured. The average height of the tissue was 15.65 μm and 5HT labeled cells were counted over the whole thickness. The optical fractionator method (Gundersen, 1986, Chakraborty et al., 2003) was used to estimate the total number of 5-HT immunolabelled neurons in the MRN or DRN which were counted as two separate categories. The neuronal number estimates did not depend on a direct measurement of the volume of reference of the region considered and therefore tissue shrinkage during histological processing would not influence the neuronal number estimates. The total cell number is determined as the number of cells counted x sampling probability^{-1} where the sampling probability^{-1} is (ssf x asf x tsf)$^{-1}$ (ssf is section sampling fraction (0.33); asf is area sampling fraction; tsf is the thickness fraction determined as the height, H, of the unbiased virtual counting spaces divided by t, the thickness of the slice after histological processing, H/t). Coefficient of error (CE) values for the cell number estimates were determined using the Gundersen method incorporated in the StereoInvestigator software and then averaged for each group.

The volumes of the MRN and DRN were measured by drawing the contour plot of each region at 10X magnification and then multiplying the contour area by the total thickness of the interslice distance (number of sections between each contour multiplied by slice thickness i.e. 3 x 40 μm). Volume estimates were calculated based on postprocessing tissues that have shrunk in all three directions. No attempts were made to correct for shrinkage because it probably differs in the z and x-y directions.

Tryptophan hydroxylase Immunohistochemistry and Microscopy

To identify whether subregions of the DRN might be affected by 13-*cis*-RA treatment we used tryptophan hydroxylase 2 (TPH-2) immunolabelling. Tissue sections from –4.84 to –4.24 mm from Bregma (Paxinos and Franklin, 2001) were selected for TPH-2 staining. All washes were performed in PBS at room temperature. Sections were washed and blocked in 3% bovine serum albumin for 1 h. Sections were then incubated with rabbit polyclonal anti-TPH2 antibody (Millipore, Billerica, MA, USA, 1:200) overnight at 4°C. Subsequently, sections were washed in PBS and incubated in donkey anti- rabbit alexafluor 568 secondary antibody (Invitrogen,1:1000) for 2 h at room temperature. After washing, sections were mounted and coverslipped using ProLong Gold (Invitrogen) and stored at 4°C prior to imaging. Images were captured using a confocal laser scanning microscope (laser λ 488nm, Zeiss LSM510, Carl Zeiss Ltd., UK) with a 10x objective. The aqueduct and the medial longitudinal fasiculus were used as anatomical borders to demarcate the DRN (Paxinos and Franklin, 2001). All TPH-2 positive cells were counted in alternate sections, in a total of 8 sections per mouse, and the estimated neuronal number reported as the total in all 8 sections counted. The subregions examined were the DRN ventrolateral part (DRVL), the DRN dorsal part (DRD) and the DRN ventral part (DRV) as identified previously by TPH-2 immunoreactivity (Lowry et al., 2008). The observer was blinded to the treatment of each group during cell imaging and counting.

Statistical analysis

Data are reported as the mean ± S.E.M of n animals and were subject to a two-tailed *t*-test to analyse the differences between vehicle treated control, and 13-*cis*-RA treated, mouse brains. The n number for each region investigated varies because of variability in the tissue quality of some of the sections.

RESULTS

To identify whether chronic treatment with13-*cis*-RA altered the number of serotoninergic cells in

the raphe nuclei, we performed a stereological analysis of immunohistochemically labelled

sections from vehicle control and drug treated adult mouse brains. Qualitative examination

revealed that no gross anatomical or histochemical abnormalities were observed in 13-*cis*-RA

treated brains compared with vehicle treated controls. 5-HT-immunolabelled cells were easily

identified in tissue sections through the MRN and DRN (Fig. 1A-D). For the DRN, the average

number of cells counted per mouse was 419.8 ± 47.2 and 458.3 ± 35.6 for control and treated

groups respectively (n=7-10, P=0.50). For the MRN, the average number of cells counted per

mouse was 153.8 ± 21.3 and 160 ± 35.6 for control and treated groups respectively (n=7-10,

P=0.76). There were no differences between vehicle control and 13-*cis*-RA treated brains in the

estimated number of 5-HT immunolabelled neurons in the MRN or DRN (Fig. 1E). In the MRN,

the estimated number of 5-HT immunolabelled cells was 1815 ± 194 in control tissues compared

with 1954 ±111 in the 13–*cis*-RA treated group (P=0.51, n=8 control, n=10 treated), estimated

with an average CE of 0.098 ± 0.006 and 0.099 ± 0.006 respectively. The number of 5-HT

immunolabelled cells was much higher in the DRN when compared to the MRN with an

estimated cell number of 7148 ± 377 in the control group and 7578 ± 424 in the 13-*cis*-RA

treated group (P=0.53, n=7 control, n=10 treated), estimated with a CE of 0.081 ± 0.011 and

0.073 ± 0.004 respectively.

Further investigation of the volume of the MRN and DRN revealed no significant

differences between vehicle control and 13-*cis*-RA treated mice (Fig. 2). The volume of the MRN

was 0.14 ± 0.02 mm^3 in the control group and 0.14 ± 0.01 mm^3 in the 13-*cis*-RA treated group

(P=0.99, n=8 control, n=10 treated), estimated with an average CE of 0.043 ± 0.005 and 0.027 ±

0.003 respectively. Similarly, in the larger DRN, the volume of the nucleus in control mice was

0.28 ± 0.02 mm^3 compared with 0.29 ± 0.01 mm^3 in 13-*cis*-RA treated mice (P=0.42, n=7

control, n=10 treated), estimated with an average CE of 0.049 ± 0.004 and 0.041 ± 0.004

respectively (Fig. 2A). Furthermore there was no difference in the density of cells contained

within the MRN or DRN between control and treated groups (Fig 2B, P=0.34 for MRN, P=0.91

for DRN, n=7-10 per group).

To exclude the possibility that subregional differences in the number of serotoninergic

neurons could be obscured in the stereological analysis, we also examined the numbers of

serotoninergic cells within subregions of the DRN using TPH-2 immunoreactivity (Fig. 3). The

number of TPH-2 immunolabelled cells in the DRV, DRD and DRVL was not significantly

different when vehicle control sections were compared with 13-*cis*-RA treated brains (Fig. 3, P=

0.69 DRV; P=0.21 DRD, P=0.35 DRVL, n=6).

Taken together these data show that chronic administration of 13-*cis*-RA does not

decrease serotoninergic cell number in the raphe nuclei of young adult mice.

DISCUSSION

In this study we have shown that chronic 13-*cis*-RA treatment has no effect on the number of serotoninergic neurons in the raphe nuclei of young adult mice compared with controls. Stereological analysis of 5-HT immunolabelled neurons revealed that the volume, number of serotoninergic neurons and neuronal density of the MRN and DRN was not affected by 13-*cis*-RA treatment. Furthermore, analysis of DRN subregions showed that there was not a significant difference in the number of serotoninergic neurons in the DRV, DRD and DRVL following 13-*cis*-RA treatment. We have previously shown that chronic treatment with 13-*cis*-RA induces an increase in depression-related behaviours (O'Reilly et al., 2006, Trent et al., 2009). In addition, we have shown that in mice treated with 13-*cis*-RA there is a functional uncoupling of the dorsal raphe nuclei and the hippocampus (O'Reilly et al., 2009). However, the data in the present study suggest that these effects of 13-*cis*-RA are not due to neuronal loss in the MRN or DRN.

To our knowledge, this study represents the first quantitative estimate of the number of serotoninergic neurons in the DRN and MRN of the adult mouse obtained by unbiased stereological methods. The total number of 5-HT immunolabelled neurons was estimated to be approximately 7500 in the DRN and 1900 in the MRN of DBA/2J adult mice. During development, at postnatal day 7 in the mouse, similar methods have shown that the number of 5-HT cell bodies in the DRN is 5641 ± 456 (Donovan et al., 2002) which is consistent with our estimates in the adult mouse. Stereological estimates of serotoninergic neuronal number in the adult DRN range from 15,000 in rats (Casu et al., 2004, Strackx et al., 2008), 95,000 in dogs (Bernedo et al., 2009) through 51,000 up to 165,000 in humans (Underwood et al., 1999, Bielau et al., 2005). This variability in the number of serotoninergic neurons likely reflects the interspecies variation in size.

Increases, decreases and no change in 5-HT cell number have all been reported in depressed human patients (Underwood et al., 1999, Baumann et al., 2002, Hendricksen et al., 2004). Our original hypothesis, that chronic treatment with 13-*cis*-RA would decrease serotoninergic cell number, was largely based on evidence that 13-*cis*-RA and other retinoids have been shown to induce apoptosis (Sun et al., 2004, Guruvayoorappan et al., 2008, Liu et al., 2009). The serotoninergic neurons of the DRN project to most forebrain areas, including those crucial for emotional behaviours, such as the amygdala and paraventricular nucleus of the hypothalamus, as well as limbic structures, such as the hippocampus (Azmitia and Segal, 1978, Imai et al., 1986, Mamounas et al., 1991, Petrov et al., 1992). An increase in cell death in the DRN in response to retinoid treatment could therefore lead to a reduction in serotoninergic input to these forebrain regions involved in mediating responses to stress. Such a decrease could account for the increase in depression-related behaviours, and the functional uncoupling of the DRN and hippocampus, observed following 13-*cis*-RA treatment (O'Reilly et al., 2006, O'Reilly et al., 2009, Trent et al., 2009). Given the proposed trophic role of serotonin (Whitaker-Azmitia, 2001), a decline in serotoninergic input to the hippocampus could also account for the reported decreases in adult neurogenesis following 13-*cis*-RA treatment, an effect which may be pro-depressive (Crandall et al., 2004). Ishikawa et al. (2008) used slice cultures from embryonic day 20 rats to show that high concentrations of 13-*cis*-RA (100 μM) decreased serotoninergic cell number. While these effects were blocked by nanomolar concentrations of RAR/RXR antagonists, indicating a receptor-mediated effect, concentrations of 13-*cis*-RA as high as 100 μM have not been achieved *in vivo*. Indeed, the chronic retinoid treatment regime we used here gives rise to plasma retinoid levels of 1.5 ± 0.4 μg/ml (5.1 μM; O'Reilly et al., 2006), well below the high concentrations used to show a decrease in serotoninergic cell number *in vitro* (Ishikawa et al., 2008). These lower plasma retinoid levels induce depression-related behaviour (O'Reilly et al., 2006) but did not reduce the number of serotoninergic neurons in the raphe nucleus

suggesting that some other, non-apoptotic, mechanism accounts for the change in depression-related behaviour.

Although the total number of serotoninergic cells arising in the raphe nuclei remain unchanged, it is still possible that the serotoninergic input to the hippocampus is impaired by chronic 13-*cis*-RA treatment. It is known that the dendritic and cellular morphology of cultured cells is affected by high levels of 13-*cis*-RA (Ishikawa et al., 2008, Liu et al., 2008) and if this is also true for the *in vivo* situation there would be a loss of serotoninergic synaptic contacts occurring within the hippocampus and a reduced serotoninergic drive into further hippocampal contacts. Not only may this directly account for the behavioural changes previously reported (O'Reilly et al., 2006, Trent et al., 2009) but it may also account for the functional uncoupling seen between the DRN and the hippocampus (O'Reilly et al., 2009). However, such changes in morphology are unlikely to occur at the plasma retinoid levels that are able to induce an increase in depression-related behaviours, as discussed above.

An alternative explanation for the functional uncoupling of the DRN from the hippocampus could be altered activity of serotoninergic neurons following 13-*cis*-RA treatment. Interestingly, mice subjected to chronic stress, a well established paradigm for inducing depressive-like behaviours, show reduced firing of serotoninergic neurons in the DRN (Bambico et al., 2009). The firing rate of serotoninergic neurons from the DRN can be regulated by 5-HT$_{1A}$ autoreceptors, where a decrease in receptor number leads to increased basal firing activity (Richer et al., 2002) and 5-HT$_{1A}$ receptor stimulation by 5-HT$_{1A}$ agonists or increased intra-cellular 5-HT conversely decreases the firing activity of these neurons (Blier et al., 1998).13-*Cis*-RA, at 2.5 and 10 μM, increased 5-HT$_{1A}$ receptor and 5-HTT protein levels and intracellular 5-HT *in vitro* (O'Reilly et al., 2007). If these changes are recapitulated following 13-*cis*-RA treatment *in vivo* one would expect the activity of serotoninergic projections from the DRN to be diminished, potentially leading to an increase in depression-related behaviour.

Acknowledgements

Figure 1. Immunohistochemical analysis of serotoninergic neurons in the brain of vehicle control and 13-*cis*-RA treated young adult mice. Illustrative photomicrographs of coronal sections from control mouse brains showing that 5-HT immunolabelled neurons were readily detected in the raphe nuclei in Nissl counter-stained tissue sections at 2x (A) and 4x (B) magnification from which contour plots were drawn. Higher magnification images, 40x (C) and 100x (D), were used for cell counting and show the representative density of 5-HT immunolabelled neurons. The number of serotoninergic neurons in the median (MRN) and dorsal (DRN) raphe nuclei were counted (E). Data shown are mean ± S.E.M.. For MRN control n=8, 13-*cis*-RA n= 10; for DRN control n=7, 13-*cis*-RA n=10.
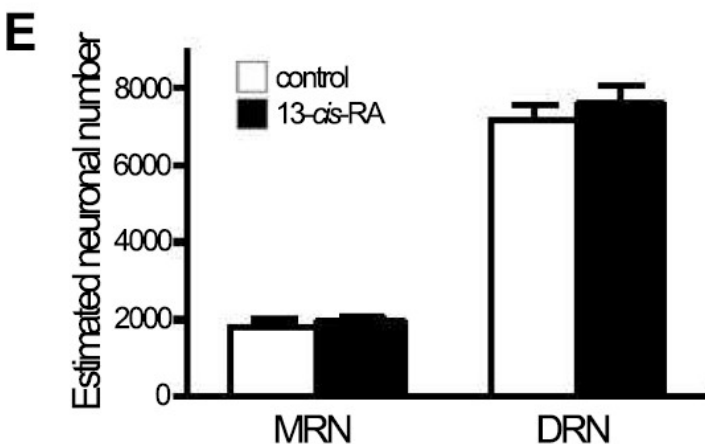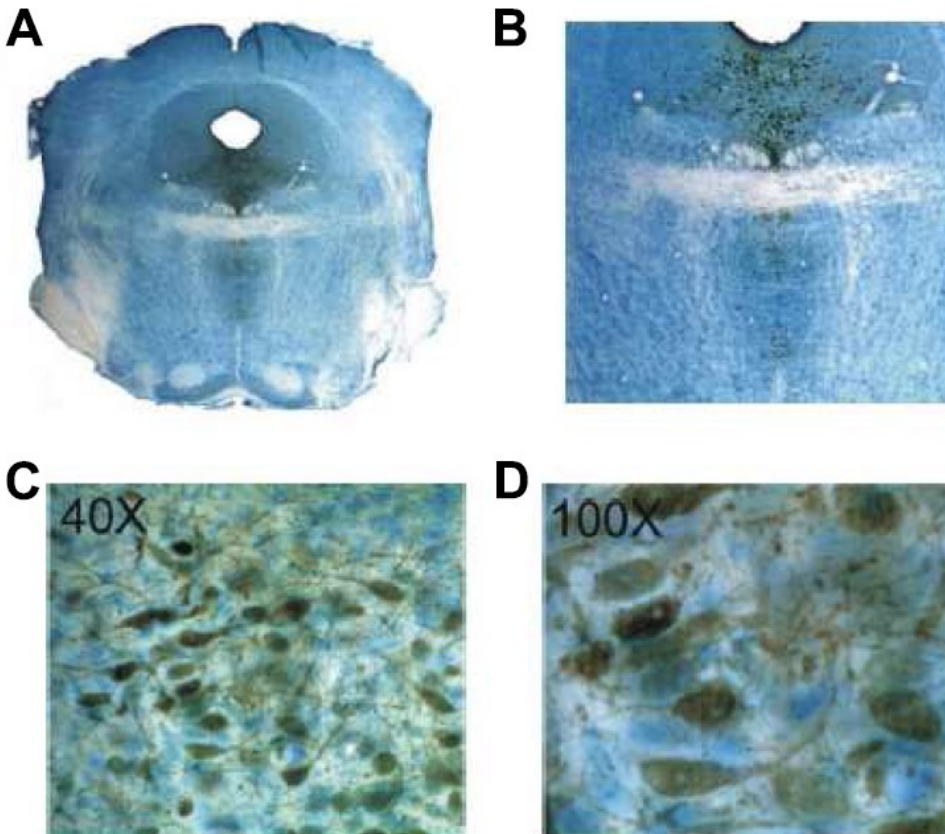
Figure 2. Quantitative analysis of the volume of the raphe nuclei in vehicle control and 13-*cis*-RA treated young adult mice. Chronic 13-*cis*-RA treatment did not alter the estimated volume (A) or the estimated density of serotoninergic neurons (B) in either the median (MRN) or dorsal (DRN) raphe nuclei compared with vehicle treated controls. Data shown are mean ± S.E.M. For MRN control n=8, 13-*cis*-RA n= 10 ; for DRN control n=7, 13-*cis*-RA n=10.
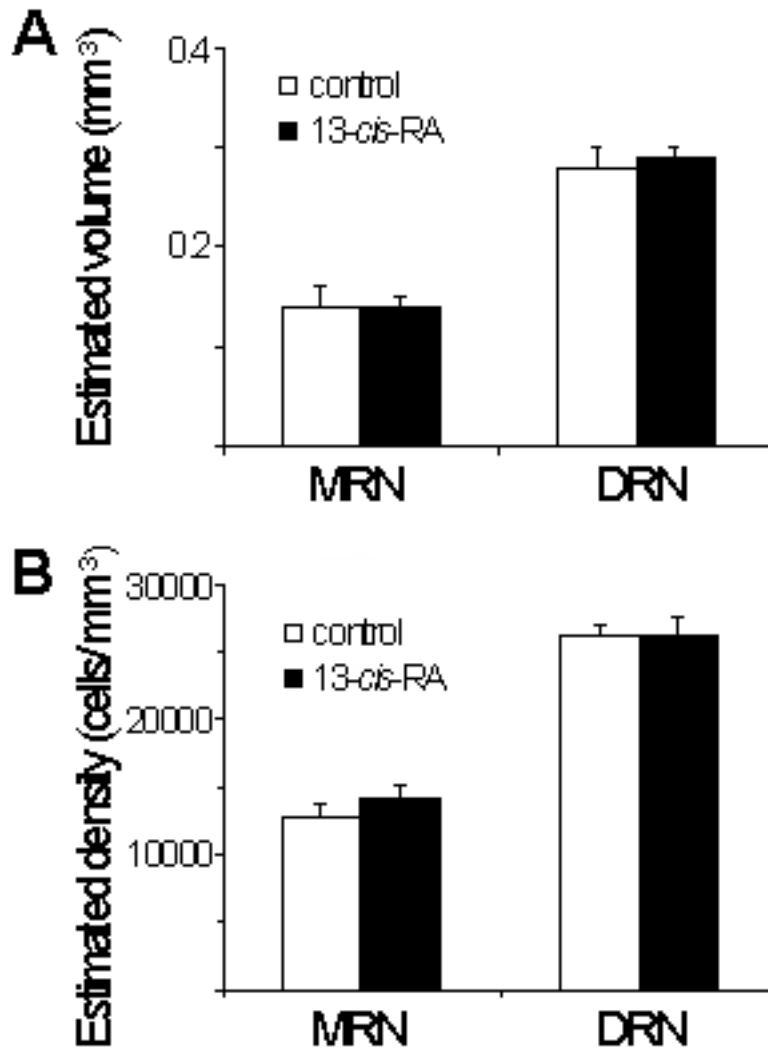
Figure 3. Analysis of TPH-2 immunolabelling in the dorsal raphe nucleus from vehicle control and 13-*cis*-RA treated adult mice. (A) Illustrative confocal micrograph showing the dorsal raphe nucleus, bounded by the aqueduct (Aq) and medial longitudinal fasciculus (mlf), and the subregions of the dorsal raphe nucleus: the dorsal raphe nucleus dorsal part (DRD), ventral part (DRV) and ventrolateral part (DRVL). Scale bar: 40 μm (B) TPH-2 immunolabelled cells were counted to estimate the number of serotoninergic neurons in each dorsal raphe subregion for both vehicle control and 13-*cis*-RA treated mice. Data shown are mean ± S.E.M., n= 6 per group.
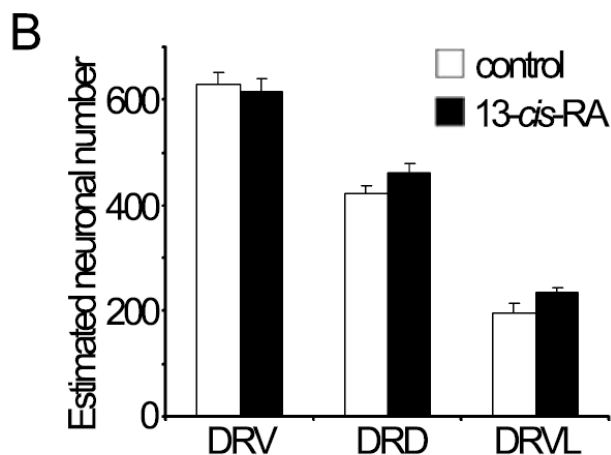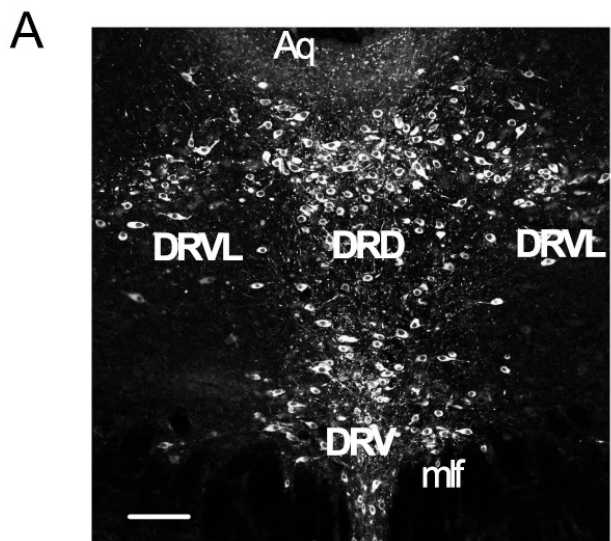
REFERENCES

Arias B, Arranz MJ, Gasto C, Catalan R, Pintor L, Gutierrez B, Kerwin RW, Fananas L
(2002)Analysis of structural polymorphisms and C-1018G promoter variant of the 5-HT(1A)
receptor gene as putative risk factors in major depression. Mol Psychiatry 7:930-932.

Azmitia EC, Segal M (1978)An autoradiographic analysis of the differential ascending
projections of the dorsal and median raphe nuclei in the rat. J Comp Neurol 179:641-667.

Bambico FR, Nguyen N-T, Gobbi G (2009)Decline in serotonergic firing activity and
desensitization of 5-HT1A autoreceptors after chronic unpredictable stress. European
Neuropsychopharmacology 19:215-228.

Barnes NM, Sharp T (1999)A review of central 5-HT receptors and their function.
Neuropharmacology 38:1083-1152.

Baumann B, Bielau H, Krell D, Agelink MW, Diekmann S, Wurthmann C, Trubner K, Bernstein
HG, Danos P, Bogerts B (2002)Circumscribed numerical deficit of dorsal raphe neurons in
mood disorders. Psychol Med 32:93-103.

Bell C, Abrams J, Nutt D (2001)Tryptophan depletion and its implications for psychiatry. Br J
Psychiatry 178:399-405.

Bernedo V, Insua D, Suárez M-L, Santamarina G, Sarasa M, Pesini P (2009)beta-amyloid
cortical deposits are accompanied by the loss of serotonergic neurons in the dog. The Journal
of Comparative Neurology 513:417-429.

Bielau H, Mawrin C, Krell D, Agelink MW, Trubner K, Davis R, Gos T, Bogerts B, Bernstein HG, Baumann B (2005)Differences in activation of the dorsal raphe nucleus depending on performance of suicide. Brain Res 1039:43-52.

Blakely RD, Berson HE, Fremeau RT, Jr., Caron MG, Peek MM, Prince HK, Bradley CC (1991)Cloning and expression of a functional serotonin transporter from rat brain. Nature 354:66-70.

Blier P, de Montigny C (1987)Modification of 5-HT neuron properties by sustained administration of the 5-HT1A agonist gepirone: electrophysiological studies in the rat brain. Synapse 1:470-480.

Blier P, de Montigny C (1994)Current advances and trends in the treatment of depression. Trends Pharmacol Sci 15:220-226.

Blier P, Pineyro G, el Mansari M, Bergeron R, de Montigny C (1998)Role of somatodendritic 5-HT autoreceptors in modulating 5-HT neurotransmission. Ann N Y Acad Sci 861:204-216.

Bremner JD, McCaffery P (2008)The neurobiology of retinoic acid in affective disorders. Prog Neuropsychopharmacol Biol Psychiatry 32:315-331.

Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, McClay J, Mill J, Martin J, Braithwaite A, Poulton R (2003)Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science 301:386-389.

Casu M, Pisu C, Lobina C, Pani L (2004)Immunocytochemical study of the forebrain serotonergic innervation in Sardinian alcohol-preferring rats. Psychopharmacology 172:341-351.

Chakraborty TR, Hof PR, Ng L, Gore AC (2003)Stereologic analysis of estrogen receptor alpha (ER alpha) expression in rat hypothalamus and its regulation by aging and estrogen. J Comp Neurol 466:409-421.

Cocco S, Diaz G, Stancampiano R, Diana A, Carta M, Curreli R, Sarais L, Fadda F (2002)Vitamin A deficiency produces spatial learning and memory impairment in rats. Neuroscience 115:475-482.

Crandall J, Sakai Y, Zhang J, Koul O, Mineur Y, Crusio WE, McCaffery P (2004)13-cis-retinoic acid suppresses hippocampal cell division and hippocampal-dependent learning in mice. Proc Natl Acad Sci U S A 101:5111-5116.

Delgado PL, Price LH, Miller HL, Salomon RM, Licinio J, Krystal JH, Heninger GR, Charney DS (1991)Rapid serotonin depletion as a provocative challenge test for patients with major depression: relevance to antidepressant action and the neurobiology of depression. Psychopharmacol Bull 27:321-330.

Donovan SL, Mamounas LA, Andrews AM, Blue ME, McCasland JS (2002)GAP-43 Is Critical for Normal Development of the Serotonergic Innervation in Forebrain. J Neurosci 22:3543-3552.

Drevets W, Price J, Furey M (2008)Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. Brain Structure and Function 213:93-118.

Etchamendy N, Enderlin V, Marighetto A, Vouimba RM, Pallet V, Jaffard R, Higueret P (2001)Alleviation of a selective age-related relational memory deficit in mice by pharmacologically induced normalization of brain retinoid signaling. J Neurosci 21:6423-6429.

Ferguson S, Cisneros FJ, Gough B, Hanig JP, Berry KJ (2005). Chronic oral treatment with 13-cis-retinoic acid (isotretinoin) or all-trans-retinoic acid does not alter depression-like behaviors in rats. Toxicol Sci 87: 451-459.

Ferguson S, Cisneros FJ, Hanig JP, Berry KJ (2007). Oral treatment with ACCUTANE does not increase measures of anhedonia or depression in rats. Neurotoxicol Teratol 29: 642-651.

Gundersen HJ (1986)Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. J Microsc 143:3-45.

Guruvayoorappan C, Pradeep CR, Kuttan G (2008)13-cis-retinoic acid induces apoptosis by modulating caspase-3, bcl-2, and p53 gene expression and regulates the activation of transcription factors in B16F-10 melanoma cells. J Environ Pathol Toxicol Oncol 27:197-207.

Hendricksen M, Thomas AJ, Ferrier IN, Ince P, O'Brien JT (2004)Neuropathological study of the dorsal raphe nuclei in late-life depression and Alzheimer's disease with and without depression. Am J Psychiatry 161:1096-1102.

Hull PR, D'Arcy C (2005)Acne, depression, and suicide. Dermatol Clin 23:665-674.

Imai H, Steindler DA, Kitai ST (1986)The organization of divergent axonal projections from the midbrain raphe nuclei in the rat. J Comp Neurol 243:363-380.

Ishikawa J, Sutoh C, Ishikawa A, Kagechika H, Hirano H, Nakamura S (2008)13-cis-retinoic acid alters the cellular morphology of slice-cultured serotonergic neurons in the rat. Eur J Neurosci 27:2363-2372.

Jacobs BL, Azmitia EC (1992)Structure and function of the brain serotonin system. Physiol Rev 72:165-229.

Kerr IG, Lippman ME, Jenkins J, Myers CE (1982)Pharmacology of 13-cis-retinoic acid in humans. Cancer Res 42:2069-2073.

Kitaoka K, Hattori A, Chikahisa S, Miyamoto K, Nakaya Y, Sei H (2007)Vitamin A deficiency induces a decrease in EEG delta power during sleep in mice. Brain Res 1150:121-130.

Krezel W, Ghyselinck N, Samad TA, Dupe V, Kastner P, Borrelli E, Chambon P (1998)Impaired locomotion and dopamine signaling in retinoid receptor mutant mice. Science 279:863-867.

Lane MA, Bailey SJ (2005)Role of retinoid signalling in the adult brain. Prog Neurobiol 75:275-293.

Lechin F, van der Dijs B, Hernández-Adrián G (2006)Dorsal raphe vs. median raphe serotonergic antagonism. Anatomical, physiological, behavioral, neuroendocrinological,

neuropharmacological and clinical evidences: Relevance for neuropharmacological therapy.

Progress in Neuro-Psychopharmacology and Biological Psychiatry 30:565-585.

Lemonde S, Turecki G, Bakish D, Du L, Hrdina PD, Bown CD, Sequeira A, Kushwaha N, Morris

SJ, Basak A, Ou XM, Albert PR (2003)Impaired repression at a 5-hydroxytryptamine 1A

receptor gene polymorphism associated with major depression and suicide. J Neurosci

23:8788-8799.

Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, Benjamin J, Muller CR, Hamer

DH, Murphy DL (1996)Association of anxiety-related traits with a polymorphism in the serotonin

transporter gene regulatory region. Science 274:1527-1531.

Liu J, Zhou R, He Q, Li WI, Zhang T, Niu B, Zheng X, Xie J (2009)Calmodulin kinase II

activation of mitogen-activated protein kinase in PC12 cell following all-trans retinoic acid

treatment. Neurotoxicology 30:599-604.

Liu Y, Kagechika H, Ishikawa J, Hirano H, Matsukuma S, Tanaka K, Nakamura S (2008)Effects

of retinoic acids on the dendritic morphology of cultured hippocampal neurons. J Neurochem.

Lowry CA, Hale MW, Evans AK, Heerkens J, Staub DR, Gasser PJ, Shekhar A

(2008)Serotonergic Systems, Anxiety, and Affective Disorder. Annals of the New York Academy

of Sciences 1148:86-94.

Maden M (2007)Retinoic acid in the development, regeneration and maintenance of the

nervous system. Nat Rev Neurosci 8:755-765.

Mamounas LA, Mullen CA, O'Hearn E, Molliver ME (1991)Dual serotoninergic projections to forebrain in the rat: morphologically distinct 5-HT axon terminals exhibit differential vulnerability to neurotoxic amphetamine derivatives. J Comp Neurol 314:558-586.

Mann JJ (1999)Role of the serotonergic system in the pathogenesis of major depression and suicidal behavior. Neuropsychopharmacology 21:99S-105S.

Maret S, Franken P, Dauvilliers Y, Ghyselinck NB, Chambon P, Tafti M (2005)Retinoic acid signaling affects cortical synchrony during sleep. Science 310:111-113.

Michelsen KA, Schmitz C, Steinbusch HW (2007)The dorsal raphe nucleus--from silver stainings to a role in depression. Brain Res Rev 55:329-342.

Neumeister A, Young T, Stastny J (2004)Implications of genetic research on the role of the serotonin in depression: emphasis on the serotonin type 1(A) receptor and the serotonin transporter. Psychopharmacology (Berl) 174:512-524.

O'Donnell J (2004)Polar hysteria: an expression of hypervitaminosis A. Am J Ther 11:507-516.

O'Reilly K, Bailey SJ, Lane MA (2008)Retinoid-Mediated Regulation of Mood: Possible Cellular Mechanisms. Experimental Biology and Medicine 233:251-258.

O'Reilly KC, Shumake J, Bailey SJ, Gonzalez-Lima F, Lane MA (2009)Chronic 13-cis-retinoic acid administration disrupts network interactions between the raphe nuclei and the hippocampal system in young adult mice. Eur J Pharmacol 605:68-77.

O'Reilly KC, Shumake J, Gonzalez-Lima F, Lane MA, Bailey SJ (2006)Chronic administration of 13-cis-retinoic acid increases depression-related behavior in mice. Neuropsychopharmacology 31:1919-1927.

O'Reilly KC, Trent S, Bailey SJ, Lane MA (2007)13-cis-Retinoic Acid Alters Intracellular Serotonin, Increases 5-HT1A Receptor, and Serotonin Reuptake Transporter Levels In Vitro. Experimental Biology and Medicine 232:1195-1203.

Paxinos G, Franklin KBJ (2001) The Mouse Brain in Stereotaxic Coordinates: Elsevier.

Petrov T, Krukoff TL, Jhamandas JH (1992)The hypothalamic paraventricular and lateral parabrachial nuclei receive collaterals from raphe nucleus neurons: a combined double retrograde and immunocytochemical study. J Comp Neurol 318:18-26.

Richer M, Hen R, Blier P (2002)Modification of serotonin neuron properties in mice lacking 5-HT1A receptors. Eur J Pharmacol 435:195-203.

Sharp T, Bramwell SR, Grahame-Smith DG (1989)5-HT1 agonists reduce 5-hydroxytryptamine release in rat hippocampus in vivo as determined by brain microdialysis. Br J Pharmacol 96:283-290.

Strackx E, van den Hove DL, Steinbusch HP, Prickaerts J, Vles JS, Blanco CE, Steinbusch HW, Gavilanes AW (2008)Fetal asphyxia leads to a decrease in dorsal raphe serotonergic neurons. Dev Neurosci 30:358-366.

Sun SY, Hail N, Jr., Lotan R (2004)Apoptosis as a novel target for cancer chemoprevention. J Natl Cancer Inst 96:662-672.

Trent S, Drew CJ, Mitchell PJ, Bailey SJ (2009)Chronic treatment with 13-cis-retinoic acid changes aggressive behaviours in the resident-intruder paradigm in rats. Eur Neuropsychopharmacol 19:876-886.

Underwood MD, Khaibulina AA, Ellis SP, Moran A, Rice PM, Mann JJ, Arango V (1999)Morphometry of the dorsal raphe nucleus serotonergic neurons in suicide victims. Biol Psychiatry 46:473-483.

Whitaker-Azmitia PM (2001)Serotonin and brain development: role in human developmental diseases. Brain Res Bull 56:479-485.

Zouboulis CC, Piquero-Martin J (2003)Update and future of systemic acne treatment. Dermatology (Basel, Switzerland) 206:37-53.