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Anterior thalamic lesions produce chronic and profuse transcriptional de-regulation in retrosplenial cortex: A model of retrosplenial hypoactivity and covert pathology

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Abstract

Anterior thalamic lesions are thought to produce ‘covert pathology’ in retrosplenial cortex, but the causes are unknown. Microarray analyses tested the hypothesis that thalamic damage causes a chronic, hypo-function of metabolic and plasticity-related pathways (Experiment 1). Rats with unilateral, anterior thalamic lesions were exposed to a novel environment for 20 minutes, and granular retrosplenial tissue sampled from both hemispheres 30 minutes, 2h, or 8h later. Complementary statistical approaches (analyses of variance, predictive patterning and gene set enrichment analysis) revealed pervasive gene expression differences between retrosplenial cortex ipsilateral to the thalamic lesion and contralateral to the lesion. Selected gene differences were validated by QPCR, immunohistochemistry (Experiment 1), and *in situ* hybridisation (Experiment 2). Following thalamic lesions, the retrosplenial cortex undergoes profuse cellular transcriptome changes including lower relative levels of specific mRNAs involved in energy metabolism and neuronal plasticity. These changes in functional gene expression may be largely driven by decreases in the expression of multiple transcription factors, including *brd8*, *c-fos*, *fra-2*, *klf5*, *nfix*, *nr4a1*, *smad3*, *smarcc2*, and *zfp9*, with a much smaller number (*nfat5*, *neuroD1*, *RXRγ*) showing increases. These findings have implications for conditions such as diencephalic amnesia and Alzheimer’s disease, where both anterior thalamic pathology and retrosplenial cortex hypometabolism are prominent.

Keywords

Alzheimer’s disease; gene; microarray; pathology; rat; thalamus

Introduction

A growing list is emerging of disorders that exhibit memory impairments associated with retrosplenial cortex dysfunction. Such disorders include Alzheimer’s disease (e.g.

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Minoshima *et al.*, 1997; Nestor *et al.*, 2003a), vascular dementia (Martinez-Bisbal *et al.*, 2004), diencephalic amnesia (Aupée *et al.*, 2001; Reed *et al.*, 2003), hypoxia-induced amnesia (Reed *et al.*, 1999; Aupée *et al.*, 2001), hypo-thyroidism (Krausz *et al.*, 2004), epilepsy (Archer *et al.*, 2003), and schizophrenia (Mitelman *et al.*, 2003; Laurens *et al.*, 2005; Newell *et al.*, 2005). In many of these cases the retrosplenial cortex exhibits a marked hypoactivity reflecting reductions in glucose utilization, regional cerebral blood flow or blood-oxygen-level-dependent activity (Minoshima *et al.*, 1997; Reed *et al.*, 1999; Aupée *et al.*, 2001; Archer *et al.*, 2003; Nestor *et al.*, 2003a; Reed *et al.*, 2003; Krausz *et al.*, 2004; Martinez-Bisbal *et al.*, 2004; Laurens *et al.*, 2005). A possible explanation of these findings is that pathology in a site directly connected to the retrosplenial cortex is sufficient to impair retrosplenial function and, thereby, disrupt memory processes.

The anterior thalamic nuclei (ATN) project densely upon the retrosplenial cortex (Vogt *et al.*, 1981; van Groen *et al.*, 1993) and there is evidence that these two regions are functionally interdependent (Sutherland and Hoising, 1993). Furthermore, pathology in the anterior thalamic nuclei is closely associated with diencephalic amnesia (Valenstein *et al.*, 1987; Aggleton and Sahgal, 1993; Harding *et al.*, 2000; Maguire 2001; van der Werf *et al.*, 2003). Likewise, in Alzheimer's disease ATN pathology is prominent at early stages (Masliah *et al.*, 1989; Braak and Braak, 1991a, 1991b) while the retrosplenial region is often the first area to exhibit metabolic hypoactivity (Minoshima, 1997; Kogure *et al.*, 2000; Desgranges *et al.*, 2002; Matsuda *et al.*, 2002; Drzezga *et al.*, 2003; Nestor *et al.*, 2003a, 2003b). Such findings suggest that anterior thalamic damage could severely disrupt retrosplenial cortex function, so linking these two regions in a shared set of neurological disorders, all displaying memory loss.

Preliminary evidence for this view comes from the finding (van Groen *et al.*, 1993) that large, unilateral thalamic lesions decrease a marker of cell metabolism, cytochrome oxidase, in granular retrosplenial cortex (R_{gb}). The thalamic lesions in that study were, however, very extensive, involving many nuclei. More direct support comes from the finding that selective ATN lesions (Jenkins *et al.*, 2004) produce a dramatic loss of the immediate-early gene (IEG) products of *c-fos* and *zif268* in R_{gb}, including a 90% reduction of c-Fos in superficial R_{gb} (Jenkins *et al.*, 2004). These changes were striking as, 1) they represented the greatest IEG loss found anywhere in these rats' brains, 2) they occurred even though many other afferents to R_{gb} were left intact, 3) they are selective as lesions to other input areas such as the entorhinal cortex, the postrhinal cortex, and the laterodorsal thalamic nucleus do not produce such IEG losses in retrosplenial cortex (Jenkins *et al.*, 2004; Poirier *et al.*, 2006). Perhaps more remarkable is the finding from a preliminary *in vitro* study that prior unilateral anterior thalamic lesions block the induction of long-term depression (LTD) in ipsilateral retrosplenial (R_{gb}) slices (Garden *et al.*, 2006). This loss of plasticity is confined to the superficial cortical laminae and, hence, in register with the IEG changes, yet the microcircuits are intact in this preparation and so the abnormality cannot be the lack of an afferent signal (Garden *et al.*, 2006). These *in vitro* data also provide strong evidence for 'covert pathology' (i.e. a functional lesion where there is no pathology detectable by standard histological means) as there is no overt pathology in the retrosplenial cortex following anterior thalamic nuclei lesions (van Groen *et al.*, 1993; Jenkins *et al.* 2004) and the IEG hypoactivity seems permanent (Jenkins *et al.*, 2004). These recent findings all suggest that the impact of anterior thalamic damage might be amplified via covert pathology in the retrosplenial cortex. Lesions were placed unilaterally in the anterior thalamic nuclei, making it possible to compare the retrosplenial cortices in the 'intact' and 'lesioned' hemispheres. This within-subjects design helped to restrict variance.

Objectives

The present study, therefore, sought to identify candidate molecular mechanisms that could instantiate permanent retrosplenial dysfunctions after ATN damage. Using microarray technology we provide the first comprehensive map of gene expression abnormalities in the retrosplenial cortex after ATN pathology. Particular questions were how ATN lesions might cause retrosplenial cortex hypometabolism and disrupt plasticity-associated functions. In Experiment 1, selected gene differences found by microarray were validated with Quantitative Polymerase Chain Reaction and immunohistochemistry. In Experiment 2, *in situ* hybridization was used to validate one further gene, *gabrd*. This gene was selected as GABA(A) activity is critical in regulating glutamatergic afferents to the cortex, such as those from ATN to the retrosplenial cortex (Gonzalo-Ruiz *et al.*, 1997; Ichinohe and Rockland, 2002; Li *et al.*, 2002).

Methods

Both experiments examined the status of the granular retrosplenial cortex following a unilateral anterior thalamic lesion. The principal data were derived from the microarray study (Exp. 1), but these findings were verified for particular genes by the use of immunohistochemistry (IHC, Exp. 1), Quantitative Polymerase Chain Reaction (QPCR, Exp. 1) and *in situ* hybridization (Exp. 2). All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and associated guidelines.

Experiment 1

Subjects—Male pigmented rats (total n = 12, Dark Agouti strain; Harlan, UK) were housed in pairs under a 13 hour light/11 hour dark cycle with *ad libitum* access to food and water. Each animal was extensively habituated to handling. The weight of the animals was monitored (212–248 g at the time of surgery).

Surgery—Unilateral lesions were placed in the anterior thalamic nuclei as the projections to the retrosplenial cortex remain ipsilateral, making it possible to have within-subject comparisons between the ‘Lesion’ (ipsilateral to the thalamic lesion) and the ‘Intact’ (contralateral to the thalamic lesion) hemispheres. Excitotoxic lesions were made with the goal of minimizing damage to fibers of passage.

Animals (n=12) were first anaesthetized with an intraperitoneal injection of pentobarbitone sodium (Sagatal, 75mg/kg), and then placed in a stereotaxic frame (David Kopf Instruments, CA). A craniotomy was made over both hemispheres. Excitotoxic lesions were produced by injecting 0.19 µl of N-methyl-D-aspartate (NMDA; Sigma Chemicals UK; 0.12 M in phosphate buffered saline (PBS), pH 7.2) into two sites in the same hemisphere using a 1 µl syringe (Hamilton, Switzerland). The stereotaxic coordinates were as follows: antero-posterior, –0.5 from bregma; medio-lateral, 1.0 and 1.7 from the midline; dorso-ventral, –6.3 and –5.7 from the top of the dura for the medial and lateral injections, respectively. The incisor bar was set at +5.0. Antibiotic powder (Aureomycin, Fort Dodge Animal Health, Southampton, UK) was subsequently applied topically and all rats also received a 5 ml subcutaneous injection of glucose saline. Paracetamol was dissolved in the rats’ drinking water, and they were observed daily until recovery. A period of six to nine weeks preceded tissue sampling.

Behavioral Procedures—Five days preceding tissue extraction, the animals were individually housed. In order to minimize unwanted disruption the rats were first habituated daily to a separate holding room in which there were no other rats. Twenty-four hours prior to tissue extraction, the animals were placed in this new holding room, under the standard

feeding regimen and light cycle. The next day, the animals were individually placed for 20 minutes in a novel, larger cage with different flooring in a novel environment of different dimensions and containing different visual stimuli. The purpose of this manipulation was to increase the likelihood of transcript expression, and so minimize floor effects.

Retrosplenial tissue from animals with unilateral anterior thalamic lesions was sampled at three time points following exposure to the novel environment (30 min, 2 and 8hrs from onset of exposure, Fig. 1a), but at comparable times of day. The three delays allowed the detection of changes in genes that are expressed early and late after stimulation (e.g. Cavallaro *et al.*, 2002; Hong *et al.*, 2004).

Anatomical nomenclature—The retrosplenial cortex of the rat (area 29) comprises two major subregions, granular and dysgranular (Vogt and Peters, 1981). The granular retrosplenial cortex can be subdivided (Vogt and Peters, 1981) into a caudal area a (Rga) and a more rostral and dorsal area b (Rgb). It should be noted that the rat brain does not possess a distinct area 23, rather the retrosplenial cortex occupies all of the posterior cingulate region. For this study, Rgb was the region of interest for three principal reasons. First, Rgb receives dense inputs from the anterior thalamic nuclei (Vogt *et al.*, 1981; van Groen *et al.*, 1993). Second, the most prominent retrosplenial IEG changes are seen in this region after anterior thalamic lesions (Jenkins *et al.*, 2004), iii) its location (immediately above the caudal half of the corpus callosum) can readily be determined in fresh tissue, so aiding both the reliability and validity of the tissue samples.

Tissue microdissection—Rats were killed between 17h and 20h by stunning followed immediately by cervical dislocation. In order to kill the rats at the same time of day, it was necessary to stage their introduction to the novel environment according to their group (8h, 2h, 0.5h prior to sacrifice, Fig. 1a). Following removal from the skull, the brain was rinsed in cold, DEPC-treated 0.1 M phosphate-buffered saline (PBS), and placed dorsal surface up in a Perspex rat brain matrix (WPI, Florida, specific for rats weighing 175-300g). The dissection lasted at most two minutes. Two 2 mm coronal slices were first obtained by making three coronal cuts (Fig. 1b), and then placed on dry ice. Discrete samples of Rgb were then punched out with a 19 gauge punch (Fig. 1c), using techniques already validated for array analysis (Holter *et al.*, 2001). The gauge size ensured that all laminae were involved while minimising any encroachment into adjacent grey or white matter.

One retrosplenial cortex punch was taken from each hemisphere for each slice. For each subject, the two punches from the same hemisphere (i.e. 'Lesion' and 'Intact') were pooled. This entire procedure was repeated for a total of 6 animals and 12 hemispheres per replicate. The punches were stored at -70°C . Confirmation of the anterior thalamic lesions used the tissue immediately anterior to the more rostral slice ('IHC', Fig. 1c). After confirmation of the lesions (see below), the tissue from two rats per time point was pooled together by hemispheric condition ('Lesion' or 'Intact').

Confirmation of anterior thalamic nuclei lesions—The lesions were confirmed in two ways. First, coronal sections from the rostral thalamus ('IHC', Fig. 1b) were processed with a Nissl stain (the anterior thalamic nuclei lie just rostral to the most anterior slice taken for Rgb tissue). Second, coronal sections from the most rostral portion of Rgb (immediately in front of the most anterior slice) were processed immunohistochemically to confirm that the thalamic lesions were sufficient to produce clear reductions of c-Fos levels in Rgb (Jenkins *et al.*, 2004).

The portion of the rat brain anterior to the most rostral coronal slice (IHC, Fig. 1c) was first placed overnight in 4% paraformaldehyde in PBS for fixation, and next again overnight in

25% sucrose in PBS. Coronal sections were cut at 40 μm on a cryostat (Leica Instruments) and three series of adjacent sections retained. One series was for Nissl staining and another for c-Fos immunohistochemistry. For Nissl staining, the sections were first mounted onto gelatine-coated slides, stained with cresyl violet, and coverslipped using DPX mountant (Fisher Scientific). For c-Fos visualization the sections were collected in 0.2 % Triton-X-100 in 0.1 M PBS at pH 7.4 (PBST). Endogenous peroxidase activity was blocked by washing the sections with 0.3 % hydrogen peroxide in PBST for ten minutes, and then four times with PBST alone for the same duration. Sections were incubated at 4°C for 48 hours in PBST with rabbit polyclonal antibody for c-Fos (1:5000, Ab-5, Oncogene Science). Sections were then rinsed for ten minutes in PBST, four times. Next, they were incubated in biotinylated secondary antibody, and then avidin-biotinylated horseradish peroxidase complex in PBST (Elite Kit, Vector Laboratories). Sections were next rinsed in Tris non-saline buffer (pH 7.4). Finally, immunoreactivity was visualized with diaminobenzidine (DAB Substrate Kit, Vector Laboratories) chromogen incubation. Sections were then mounted on gelatinised slides, dehydrated through a series of alcohol gradients and coverslipped.

Sections were viewed on a Leica DMRB microscope, digitally photographed using an Olympus DP70 camera. Counts of c-Fos-positive cells used the program analySIS^{AD} (Olympus, UK). The threshold was set at the same level for all sections and counts were made in a frame area ($1768 \times 1331 \mu\text{m}$, using 5 $\times$ magnification) that included all Rgb lamina. Counts were typically taken from three consecutive sections from both hemispheres of the retrosplenial cortex (approximately between -2.56 and -3.14 from bregma according to Paxinos and Watson, 1997), and these counts were averaged to produce a mean. A two-way paired-samples t-test compared c-Fos counts between the 'Intact' and 'Lesion' hemisphere.

RNA extraction and microarray hybridization: RNA was extracted (as described by before by Chomczynski and Sacchi, 1987) from the pooled tissue (cf. Humphries *et al.*, 2002). Briefly, the sample was homogenised in the denaturing solution (guanidinium isothiocyanate/mercaptoethanol) and then sodium acetate, phenol, and chloroform-iso-amyl alcohol mixture were added and the solution shaken vigorously. After 10 minutes incubation on ice, samples were centrifuged for 10 minutes at 14000rpm at 4°C. At this point the RNA was present in the aqueous phase. This phase was removed and mixed with ethanol and placed at -20°C for an hour for the RNA to precipitate. This solution was centrifuged again for 10 minutes and the resulting pellet was dissolved in the denaturing solution and then precipitated with ethanol at -20°C for 1 hour. After a further 10 minute centrifugation, the pellet was washed in 95% ethanol, dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water. Yields of total cellular RNA extracted from the pooled punches in each sample ranged from 1.40 – 2.52 μg . A 3 μl aliquot (approx. 0.6 μg) of each sample was supplied to the Wales Gene Park Affymetrix GeneChip Expression Profiling Service, Cardiff University. Before further processing, each RNA sample was quality controlled using Agilent RNA6000 chips. Following this check, 100ng of each sample was used to amplify biotinylated cRNA targets using Affymetrix GeneChip protocols and reagents. The biotinylated cRNA (target) was probed with rat genome 230A GeneChips. Hybridization and washing was performed using a GeneChip fluidics station 400 (Affymetrix). After scanning the microarrays, initial data processing was conducted with Microarray Suite 5.0 (Affymetrix). The average signal intensity of each array was scaled to 100. This procedure normalizes the raw data and corrects for technical variation between the arrays (e.g. differences in hybridization conditions).

QPCR validation of microarray candidate genes—QPCR was used to verify the microarray findings for nine genes. These candidate genes were chosen from different

classes of genes identified by the microarray analyses (see below). Using material from the same subjects as for the microarray analyses, for each sample 0.5 µg of total RNA was processed in a reverse transcription reaction in either the presence (RT+) or absence (RT-) of reverse transcriptase (Superscript II protocol, Invitrogen). Then 15 µl of each cDNA was diluted with 135 µl of 10 µg/ml tRNA. QPCR analysis of transcript levels was performed according to established protocols (Sugden, 2003). Briefly, QPCR was performed using a LightCycler™ 1.2 (Roche, UK) in a 10 µl volume using sense and anti-sense primers (0.5 µM; sequences given in Supplemental Information) and the dNTPs, *Taq* DNA polymerase and reaction buffer provided in the QuantiTect SYBR Green kit (Qiagen, UK). All QPCR assays used an initial 15 min., 95°C step to activate *Taq* polymerase, followed by 35-40 cycles of denaturation 95°C, 15 sec., annealing 56°C, 20 sec. and extension 72°C, 10 sec. The fluorescence of the accumulating product was acquired each cycle after an additional 3 sec. step to 3°C below the product *T_m*. All assays included a tRNA and RT negative control. Target amplification was verified for all genes as all assays gave a single melting peak and a single band of the expected size on agarose gel/EtBr electrophoresis (2.2% w/v). The standards used for quantification of copy number in cDNA samples were gene specific PCR products purified by agarose gel/EtBr electrophoresis and then quantitated by densitometry against a known quantity of a low molecular weight (109 bp) DNA marker. Standards were diluted to give 10¹ to 10⁷ copies/assay, aliquoted, stored at -80°C and used in each assay. All assays were highly efficient (>92%) and standard curves were all linear from 10¹ to 10⁷ with correlation coefficients (*r*²) >0.9990 (see QPCR Assay Characteristics in Supplemental Information)

Analyses of microarray data—Analyses used GeneSpring™ software (Version 6.1, Silicon Genetics, Agilent, Santa Clara, CA) to characterize mRNA changes in area Rgb following anterior thalamic lesions. The data for all of the analyses were first log transformed. The hybridization intensity was normalized per chip to the 50th percentile (0.01 cut-off value) and per gene to the median. Data filtering was applied using parameters recommended by Silicon Genetics. Genes that were labeled as “absent” based on their absolute signal level in all samples were removed.

In order to augment the replication reliability, the cross-gene error model was applied to the analyses of variance. Calculations for all samples thus relied on the combined within-sample and between-sample variation, taking into account replicate variability, using values produced by the scanning software for the signal precision of each transcript. A control strength cutoff value was thus calculated for “reliable” data and used as a filter for the signal strength of the probes in each condition. The cross-gene error model used by GeneSpring™ generally yields more conservative results than signal strength compared to control.

Analysis of variance: We initially used the widespread approach of fold-change analyses (see below). Lists of genes exhibiting 1.8 fold-change in expression between treatment groups were created for each time condition and then pooled. The application of parametric statistical measures for microarray data using Affymetrix chips is suitable as it has been found that such data sets exhibit good correlation with normality (Wang *et al.*, 2001; Giles and Kipling, 2003). A two-way ANOVA using Surgical Treatment (‘Intact’ and ‘Lesion’) and Time (30 min, 2h, 8h after the beginning of novel context exposure) was performed, followed by one-way ANOVAs for individual Time levels. Correction for multiple testing was applied using a false discovery rate of 0.05 (FDR, Benjamini and Hochberg, 1995), which means that 5 % of the genes that pass the statistical test can be considered to be false positives, i.e. genes that pass the test by chance.

Expression pattern cluster analysis: The Quality Threshold (QT) clustering method uses a set of rules to retain only clusters of a good quality. Only those genes for which expression

profiles are within a user-specified level of similarity will be clustered (Minimum cluster size 5; minimum similarity 0.9; similarity measure Pearson correlation). All other genes remained unclassified.

Treatment profiling: The analyses of variance were complemented by using the class prediction function of GeneSpring™. While an ANOVA on differences in gene expression levels based on fold-change may allow the production of a reasonable list of genes affected by the manipulation, the fold-change analysis is based on an arbitrary value (here, 1.8) that has no physiological correlate. For some transcripts, changes smaller than the chosen value (1.8) are biologically meaningful, so it is possible that subtle but robust findings may be hidden by such an analysis (Murphy, 2002; Pavlidis, 2003).

The class prediction function of GeneSpring™ allows the statistical comparison of gene expression levels between treatments, without any arbitrary fold-change threshold. Only those genes whose relative expression level correctly predicted class membership (e.g. treatment condition) were identified, based on robust differential expression levels. In other words, the relative expression level of these genes could predict treatment in any condition of either duplicate.

The class prediction function uses Fisher's exact test to assign a p -value for the possibility of transcripts being located by chance closer to either of the classes (according to hypergeometric distribution), on either side of a decision cut-off value. This value is created by producing for each transcript the ratio of the p -values from each class. The default cutoff value is 0.2, meaning that the magnitude of the difference between the p -values must be five or more. This forms the basis for the prediction strength value, according to which the transcripts are then ranked. A final filter was applied, limiting the number of regulated genes for further analysis to the top two percent. The class prediction analysis yielded a set of genes that always correctly identified surgical treatment membership.

Pooling the Surgical treatment conditions and producing gene class predictions for Time was unreliable as this yielded numerous group prediction errors. In contrast, pooling across time points and using the class prediction tool for the Surgical treatment variable yielded no prediction errors for the transcriptome profiling of each surgical treatment, and so only these results are presented.

Expression pattern cluster analysis: A QT Cluster analysis was conducted on this class prediction list. The parameters were the same as for the fold change analyses (minimum cluster size 5; minimum similarity 0.9; similarity measure Pearson Correlation). To identify transcripts only labeled as Expressed Sequence Tags (ESTs), we used the Basic Local Alignment Search Tool (Altschul *et al.*, 1997), a nucleotide sequence similarity search program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Transcripts that were labeled by GeneSpring™ as "similar to" a known gene were also thus confirmed. A positive identity was inferred when high similarity ratings were produced, where the possibility that a similar nucleotide sequence match would be found by chance was low (i.e. the E-value was close to zero).

Where possible, gene transcripts were next annotated using gene ontology information from the Rat Genome Database (www.rgd.mcg.edu), Genecards (www.genecards.org) and literature searches on Pubmed (www.ncbi.nlm.nih.gov/entrez/). In order to capture the overall nature of the changes produced by the thalamic lesions the genes were then classified in terms of their cellular localization and their functional role according to the Kyoto Encyclopedia of Genes and Genomes scheme (<http://www.genome.jp/kegg/>).

Transcription factor binding site analysis: The evaluation of the potential transcription binding sites shared by the promoter regions of genes identified by the fold-change and treatment profiling microarray analyses used the Match™ program (Kel *et al.*, 2003) with the TRANSFAC database (<http://www.gene-regulation.com/pub/programs.html#match>).

Gene set enrichment analysis (GSEA): We applied GSEA as another method of supervised computational data processing. Similarly to the treatment profiling approach described earlier, GSEA compared the two surgical conditions. However, instead of being based on analyses of individual genes, the strength of GSEA lies in the evaluation of the over-representation of pre-defined gene sets that are pathway-related (Subramanian *et al.*, 2005). This approach enabled a formal consideration of pathways in relation to the observed gene expression changes, and was applied as described by Subramanian and co-workers (Subramanian *et al.*, 2005).

Experiment 2

GABAergic signals are central to intrinsic retrosplenial cortex signal integration (Ichinohe and Rockland, 2002; Li *et al.*, 2002) and for the control of the feed-forward communication in thalamocortical circuits (e.g. Daw *et al.*, 2007). In light of the vital role of cortical GABA, the expression levels of the microarray-derived *gabrd* transcript were tested using a different technology, *in situ* hybridization. This method required a different group of rats.

Subjects—Male rats (n=10, Dark Agouti strain, Harlan, UK) weighing between 200 and 225 g at the time of surgery were housed in pairs under a 13 hour light/11 hour dark cycle with *ad libitum* access to food and water. Each animal was extensively habituated to handling.

Tissue preparation—For the *in situ* hybridization experiment, unilateral anterior thalamic lesions were produced by infusing 0.20 µl of 0.12 M NMDA twice, per hemisphere (for coordinates see Exp. 1). Four weeks after surgery, the rats were killed 30 min after exposure to a novel environment by CO₂ exposure and decapitation. Whole brains were rapidly removed and frozen on dry ice, then stored at –70°C until sectioned. Sections (14 µm) were cut on a cryostat (Leica Instruments) and thaw-mounted onto poly-L-lysine (hydrobromide; molecular mass >300,000; Sigma, Poole, UK)-coated glass slides (0.02 mg/ml DEPC-treated water). The sections were air-dried for 30 min, fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 5 min, rinsed in PBS for 1 min, delipidated in 70% ethanol for 4 min, and stored in 95% ethanol at 4°C.

In situ hybridisation—A DNA antisense probe complementary to nucleotides 119–159 of the rat *gabrd* gene (Shivers *et al.*, 1989; NCBI accession number NM 017289) was synthesized commercially (Sigma-Genosys, Cambridge, UK). This oligonucleotide was 3' end-labelled with [α -³⁵S]dATP (1200 Ci/mmol; New England Nuclear, UK) in a 30:1 molar ratio of radio-labelled ATP:oligonucleotide using terminal deoxynucleotidyl transferase (Promega) as described previously (Wisden and Morris, 1994). Specific activity of the ³⁵S-labelled probe was between 1.8 × 10⁵ d.p.m./microlitre probe. To define nonspecific hybridization, adjacent slide-mounted sections were incubated with the radiolabelled *gabrd* oligonucleotide probe in the presence of an excess (100×) concentration of unlabelled oligonucleotide probe.

After hybridisation, sections were opposed to Eastman Kodak (Rochester, NY) BioMax X-ray film for 2 weeks. After obtaining appropriate x-ray film exposures, sections were dipped in K5 photographic emulsion (Ilford, UK). Sections were exposed for 18 weeks at 4°C prior to development and then counterstained with 0.01% thionin.

Silver grain density was assessed in laminae II (superficial) and V (deep) of Rgb from typically three adjacent sections (−2.8 to −6.0 mm from bregma) using LeicaQWIN imaging software (Leica Microsystems). Grains (total and non-specific) were counted over 8-10 cells per hemisphere for each lamina, and the mean number of silver grains per cell calculated. The specific grain count mean was then obtained by subtracting the non-specific from the total count means. Statistical analyses (SPSS 14.0, Chicago) started with a two-way ANOVA with the factors surgical treatment and lamina. *Post hoc* tests were made where appropriate. The grain counts were obtained by an investigator blind to the treatment of each hemisphere.

Results

Experiment 1

Histology – Nissl confirmation of thalamic lesions—All animals included for the analyses exhibited discrete lesions in the anterior thalamic nuclei, removing most of the cells in the anterodorsal and anteroventral nuclei (Fig. 2). In the smallest of these 12 lesions there was some light anterior ventral nucleus sparing throughout, though mainly in the rostral part of the nucleus with less sparing further caudally. Complete loss of the anterodorsal nucleus was seen in this case, but little damage was seen in the anterior medial nucleus or the lateral dorsal nucleus. The largest lesion of the 12 cases removed all of the anterior ventral and anterior dorsal nuclei. There was damage in the anterior medial nucleus but it was not completely removed. Very limited cell loss was sometimes observed in the immediately adjacent part of the dentate gyrus, i.e. in the most rostral part of the medial blade of the dentate gyrus (n=8). The fornix always contained two needle tracks. There was also restricted damage to the rostral pole of the lateral dorsal nucleus in all cases. As expected, counts of Nissl-stained cells in the retrosplenial cortex failed to find evidence of a difference between the ‘Lesion’ and ‘Intact’ hemispheres (paired samples t-test, $t_{(11)}=1.3$, $p=0.2$; Fig. 3c).

Histology – Immunohistochemical confirmation of thalamic lesions—Further confirmation of the effectiveness of the lesions came from the expected (Jenkins *et al.*, 2004) striking decrease in the number of c-Fos-positive cells in Rgb in the ‘Lesion’ hemisphere [paired samples t-test (two-way), $t_{(11)}=6.3$, $p<0.0001$, Fig.3].

Microarray analyses—The normalized probe signal intensity profiles of individual samples were similar and normally distributed, and thus no set was removed (see Fig. S1 in supplemental material). All samples were also highly correlated ($>.90$, .25 weighing coefficient). The data quality filters yielded 9075 satisfactory ‘transcripts’ out of the original 15923 probe sets. As all comparisons were between the ‘Intact’ and the ‘Lesion’ hemisphere, the transcript analyses are based on relative and not absolute levels.

Fold-change ANOVA: A total of 319 transcripts were differentially expressed at the 1.8 fold-level. The 2-way ANOVA based on this gene expression fold-change produced a list of 32 genes exhibiting significant changes in expression levels between the surgical conditions (see Table 1), but there was no significant effect of post-novelty Time interval or significant interaction between Time and Surgical Treatment. All genes were significant for Surgical Treatment ($p<0.05$). In this list, according to the chosen false discovery rate, 1.6 genes would be expected to be false positives. Typically, the 32 genes were expressed at a relatively higher level on the ‘Intact’ than the ‘Lesion’ side (25 vs. 7 genes, respectively). Among the genes showing a 1.8 fold change were a number of transcription factors (c-fos, klf5, fra-2, zfp91), frequent functional associations being with cellular processes and genetic

information processing, most prominently relating to immune responses and the regulation of transcription.

While there was no overall effect of post-novelty time interval on gene expression, the cluster analysis on this list yielded sets of genes exhibiting three major temporal patterns expression patterns according to condition (see Table 1). A one-way ANOVA identified the time points at which 1.8 fold changes in expression levels were seen for each gene between the two surgical treatment conditions (see Table 1). The complementary nature of the profiles of these cluster analyses helps to explain why there was a null effect of post-novelty interval overall. Furthermore, while there were no systematic differences in the post-lesion durations for the three novelty context – intervals, variability in this measure could have increased the likelihood of a null result.

Treatment transcriptome profiling: The class prediction analysis yielded a list of 202 genes (see Table 2 in supplemental material) for which relative expression levels were consistent for data across all time points and both replicates, and so exhibited significant differences according to this method, e.g. a specific transcript was always significantly greater in one level of the Surgical treatment, for all time points and across both replicates. Over three quarters of the 202 genes identified by this method were at greater expression levels in the ‘Intact’ than ‘Lesion’ Rgb tissue (157 vs, 45, respectively). The cluster analysis again allowed us to further segregate genes on this list into sets according to their expression patterns across the time points for each condition (see Table 2 in supplemental material).

Both the fold change and the predictive patterning analyses specified that amongst all cellular compartments, genes found in the nucleus and the membrane appeared to be most affected. Furthermore, transcription emerged as one of the functions most frequently represented in both the fold-change and the predictive patterning analyses. In addition to the expected fall in the expression of *c-fos*, based on previous immunohistochemical studies of c-Fos protein levels after anterior thalamic lesions (Jenkins *et al.*, 2004), differences between the ‘Intact’ and the ‘Lesion’ hemispheres were also found for the transcription factors *fra-2*, *zfp91* and *klf5* based on fold-change differences. The transcription factors identified by predictive patterning included *klf5*, *brd8*, *nfat5*, *nfix*, *nr4a1*, *smad3*, *NeuroD1*, *SMARCC2*, and *RXRγ*, with *klf5* common to both methods. It is evident that diverse functions were disturbed by the lesion, affecting more pathways than only those that converge on the *c-fos* promoter region. The computational analysis of the gene promoter elements identified candidate transcription factor binding sites that were shared by the genes highlighted by the treatment profiling and the fold-change approaches. In descending order, the most common transcription factor binding sites were for FoxD3, Pax-4, Oct-1 and AP-1 for the treatment profiling, and FoxD3, Oct-1 and AP-1 for the fold-change gene lists (cf. Fig. S2 in supplemental material). AP-1, but not FoxD3, Pax-4, or Oct-1 was found to be differentially expressed between the surgical conditions. Two of the heterodimeric AP-1 binding partners, *c-fos* and *fra-2*, were lowest in the ‘Lesion’ hemisphere in the fold-change list (cf. Table 1). It is thus plausible that these two transcription factors play an important role in the regulation of the altered retrosplenial cortex response.

Both analyses yielded candidates underlying biological function changes, and we have no *a priori* reason to believe that the results of either type of analysis are the more important. Thus, in order to simplify the description of the data, the results of the fold-change and the treatment profiling analyses are integrated as they indicate many similar alterations in gene expression.

Evidence was found of widespread alterations in the expression of genes involved in a multitude of biological functions (cf. Fig. 4). These alterations are most likely chronic as

acute recovery processes such as an *increase* in c-Fos protein are thought to peak 3-10 days after injury and return to baseline before one month (Nieto-Sampedro *et al.*, 1982; Buytaert, 2001; Mingorance *et al.*, 2005). In the present study the changes included potential differences in neuroendocrine activity (e.g. *hsd11b1*, *RXRγ*, *brd8*, and *adcyap1*), cell adhesion, growth, and reorganization (e.g. *cyclinD2*, *klf5*, *lsamp*, *neuroD1*, *nptx2*, *reprim0*, *actin alpha-1*, *cbln1*, *lsamp*, *map2*, *mmp9*, *mmp24*, *myosin Ie*, *myosin IXa*, *nelf*, *nefh*, *netrin1*, *netrin-g1a*, *neurexin1*, *neuritin1*, *plexinb2*, *scn1b* and *tuba4*), as well as immune functions and inflammation (e.g. *CD74*, *CD83*, *cyslt1*, *hla-dmb*, *LOC171412*, *masp1*, *ppib*, *RT1.B beta 1*, *RT1-Da*, *tore*). The focus will be on gene expression changes reflecting energy metabolism and neuroplasticity, as these best address the goals of the experiment.

Energy production—Expression differences were found that suggest disturbances in blood, oxygen, and energetic activities (cf. Fig.5). The ‘Lesion’ hemisphere contained lower levels of genes associated with effective hemoglobin oxygen transport (*hba1*, *urod*) and glucose transport and metabolism (*exoc7*, *ehd2*, *pank4*; Ishiki and Klip, 2005; Yunfeng *et al.*, 2005). Additionally, the higher expression of *COX1/Ptgs1* after the lesion may be associated with this probable alteration in oxygenation and energy as this molecule is an important mediator of neurovascular coupling and vasodilation (e.g. Takano *et al.*, 2006). Pervasive alterations were found in mitochondrial genes, which play an important role in oxidative phosphorylation and can affect the glutamine-glutamate cycle. Deficient metabolic function is suggested by the finding that mitochondrial transcripts exhibited altered expression patterns in all mitochondrial complexes, and were almost exclusively lower in the ‘Lesion’ condition (*ndufv2*, *ndufa8*, *ndufb6*, *CoQ9*, *UCR/QCR9*, *cox6a*, *atp5o*, *atp6s14*, *mdh2*, *sdhc*, but not *cox6b*, cf. Table 2 in supplemental material, and Fig. 5). GSEA replicated this finding as gene sets for the electron transport chain (False Discovery Rate, FDR=0.044) and oxidative phosphorylation (FDR=0.040, cf. supplemental material) were both found to be relatively enriched in the ‘Intact’ condition. In fact, these were the only enriched sets having a FDR lower than 0.05 in that condition.

c-Fos and the AP-1 complex can translocate to the mitochondrion, and there are AP-1-like binding sequences in mouse mitochondrial DNA (Ogita *et al.*, 2002; Ogita *et al.*, 2003). Computational analyses of transcription factor binding sites for the genes altered in the current study identified potential AP-1 binding sites for a number of mitochondrial genes, including *ndufv2*, *ndufa8*, *COQ9*, *atp6s14* and *mdh2* (genes that were all reduced on the lesioned side). While plausible, the possibility that the deficit in c-fos expression may be directly linked to the mitochondrial deficits could not be tested in the present study.

Neuroplasticity—Genes found to be differentially regulated in this study have been shown to play a role in neuroplasticity. For example, the activity of certain transcripts that were found in lower abundance on the ‘Lesion’ side have been reported to be associated with long term potentiation [LTP, e.g. *adcyap1* (Otto *et al.*, 2001; Matsuyama *et al.*, 2003), *carbonic anhydrase 4* (White and Platt, 2001), *mmp9* (Nagy *et al.*, 2006), *ncs-1* (Genin *et al.*, 2001; Brackmann *et al.*, 2004), *neuritin1* (Wibrand *et al.*, 2006), *ppp1r1a* (Allen *et al.*, 2000)], or long term depression [LTD, e.g. *cbln1* (Hirai *et al.*, 2005), *Nr4a1* (Lindecke *et al.*, 2006), *ppp1r1a* (Mulkey *et al.*, 1994; Morishita *et al.*, 2001; Jouvenceau *et al.*, 2006), *Prkar1b* (Brandon *et al.*, 1995)]. In contrast, higher levels were found on the ‘Lesion’ side for *nptx2*, which is LTP-related (Wibrand *et al.*, 2006), and *RXRγ*, a gene reported to be involved in LTD, but not LTP (Chiang *et al.*, 1998).

Cell signaling—Cell signaling appears to be grossly affected by the lesion. The tissue in the ‘Intact’ cortex displayed greater quantities of transcripts associated with synaptic signaling (e.g. *scamp1*, *syt5*, *napa*, *ap2m1*, *ncs-1*, *exoc7*, *lrp3*, *neurexin1*, but lower for

ehd2). Neuronal signaling from the anterior thalamic nuclei to the retrosplenial cortex relies on glutamatergic inputs (Gonzalo-Ruiz *et al.*, 1997) and their interaction with GABA interneurons within the cortex (Ichinohe and Rockland, 2002). It is thus not surprising to find evidence of altered regulation of both neurotransmitters.

While no glutamate receptors were found to be differentially regulated, several transcripts suggest a disturbance in glutamatergic functions (cf. Fig. 5). An immediate-early effector gene, *nptx2*, reported to be involved in glutamate receptor clustering selectively on interneurons (O'Brien *et al.*, 1999; Mi *et al.*, 2002) was found in greater abundance on the 'Lesion' side, hinting at potentially more intense excitatory activity on these cells in this condition. Enzymes involved in the metabolism of glutamate itself and of molecules involved in glutamate functions were also affected by the anterior thalamic nuclei lesion, and likewise point an upregulation in glutamatergic activity. NMDA receptor antagonism leads to increases in glutamine synthetase and also in extended tricarboxylic acid cycling (Brenner *et al.*, 2005). Accordingly, NMDA hyperactivity may be associated with a reduction in glutamine synthetase and in a decrease in tricarboxylic acid cycling. In the present experiment, the finding of a lower expression on the 'Lesion' side of gene transcripts for glutamine synthetase, malate dehydrogenase and succinate dehydrogenase is consistent with reduced activity of the tricarboxylic acid cycle, and a potential increase in NMDA agonism.

Relatively lower levels in the 'Lesion' hemisphere of glutamine synthetase, as well as glutaminase and asparagine synthetase suggest an alteration in glutamate metabolism and homeostasis. Glutamate is converted to glutamine by glutamine synthetase, a glial-specific enzyme (Norenberg and Martinez-Hernandez, 1979), and as such, the disparity between the surgical conditions may reflect differences in mechanisms of glutamate signaling and metabolism, including neuron-glia interaction (cf. Hertz and Zielke, 2004; Patel *et al.*, 2005).

Evidence of perturbations in glutamate function also arises from apparent alterations in kynurenine metabolism. Transcripts for *cyclooxygenase 1 (COX1/ptgs1)* and *kynurenine 3-monooxygenase (KMO)*, two enzymes associated with the metabolites of this pathway, were at relatively higher levels on the 'Lesion' side. The endpoints of kynurenine metabolism are kynurenic acid and quinolinic acid (Moroni *et al.*, 1999). Kynurenic acid, a neuroprotective molecule (Carpenedo *et al.*, 2002), and quinolinic acid are respectively antagonists and agonists of NMDA receptors. Reduced levels of KMO produce an increase in kynurenic acid (Carpenedo *et al.*, 2002; Erhardt and Engberg, 2002). *COX1* has been reported to be associated with NMDA activity and the modulation of kynurenic acid levels. NMDA activation increases levels of this enzyme (Pepicelli *et al.*, 2005), and similarly, *COX1* reduction increases kynurenic acid levels (Schwieler *et al.*, 2005).

Finally, *adenylate cyclase activating polypeptide 1* increases the re-uptake of glutamate by glia (Figiel and Engele, 2000). A lower level of *adcyap1* on the 'Lesion' side could be associated with greater glutamatergic activity in this hemisphere. This observation and the evidence presented above converge to indicate a disturbance in glutamatergic signalling, potentially associated with an increase in NMDA activity in Rgb.

Transcripts for various other neurotransmitter receptors and enzymes, in addition to several sodium and potassium ion channels, appear to display robust, consistent differences between the surgical treatment conditions. Such changes were apparent for GABAergic signaling [e.g. *gabrd* and *ubqln1* (a mediator of GABA receptor subunit composition, cf. Bedford *et al.*, 2001)], and also adrenergic (*adrb3*, *adrbk1*) and serotonergic (*HT2C*). Transcripts for *HT2C* were lower on the 'Lesion' side, consistent with previous findings for *5HT1B*, those

for *Adrb3* were relatively higher on the ‘Lesion’ side, opposite to findings for *Adrb2* (van Groen *et al.*, 1993).

QPCR validation of candidate genes—Figure 6 shows the total number of transcripts in a 2 μ l volume, normalized to β -actin, for the mRNA of nine pre-selected genes. The QPCR results were highly consistent with the microarray results, strengthening confidence in the overall findings of the microarray analyses. All of the gene time point values, except for one (see below), revealed relative patterns of expression that were similar to those found by the microarray analyses (26 out of 27). The sole exception was *zyxin* at the 30 min time point (Fig. 6).

Experiment 2

In situ hybridization was used to validate the microarray data for one selected gene *gabrd*, which was reduced in the ‘Lesion’ hemisphere. This gene was chosen as GABA(A) activity is critical in regulating glutamatergic afferents to the cortex (e.g. Daw *et al.*, 2007).

Results—The unilateral anterior thalamic lesions (n=10, largest and smallest depicted in Fig. 7a) were very similar to those described for Experiment 1, although there were on average very slightly larger. In Experiment 2, the lesions included most of the cells in the anterodorsal and anteroventral nuclei, while sparing some of the anteromedial nucleus. Cell loss was found in the most rostral portion of the laterodorsal nuclei (n = 5). There was also some restricted cell loss in the immediately adjacent parts of the dentate gyrus and CA3 regions in the cases with the largest lesions. Injection tracts passed through the fornix in all cases.

Once more, there was no interhemispheric difference in counts of cresyl-stained material in Rgb after the unilateral thalamic lesions (data not shown). Although there was no overall effect of Surgical Treatment on for *gabrd* grain counts ($F_{(1,6)} = 0.23$; $p=0.65$; Fig. 7c) there was a Lamina by Surgical Treatment interaction ($F_{(1,6)} = 10.69$; $p<0.05$). Follow-up comparisons (Bonferroni adjusted) revealed significantly reduced *gabrd* grain counts in the superficial lamina (II) of the lesioned hemisphere ($p<0.05$), but there was no interhemispheric difference for lamina V ($p=0.24$).

Conclusions

- The sensitivity of the retrosplenial cortex to damage in one of its key afferents, the anterior thalamic nuclei, extended to many functions, pervading cellular activities represented in the transcriptome.
- The transcriptome changes were validated by successfully replicating several findings using a different technique, QPCR.
- Important groups of essential function exhibiting altered transcription patterns included:
 - Energy and oxygenation;
 - Neuroplasticity;
 - Cell signaling, from synaptic to intracellular communication.
- Transcription factors that exhibited expression level changes were not restricted to c-Fos or AP-1 dimer members. These transcription factors could play an important role in mediating the particular retrosplenial cortex response to anterior thalamic nuclei lesions.

- Computational analyses revealed that AP-1 is one of the most common transcription factor binding sites on those genes affected by the lesion, and proved to be the most common transcription factor that was itself (via *c-fos*) also affected by the lesion. This situation can be contrasted with the transcription FOXD3, which exhibited the most numerous binding sites on those genes affected by the distal lesion, yet its own expression levels were not affected.
- The greater sensitivity of the superficial laminae of the retrosplenial cortex to anterior thalamic lesions was reflected in a lamina-specific reduction of a specific GABA receptor subunit.

Discussion

Retrosplenial cortex dysfunction following lesions of the anterior thalamic nuclei was globally analyzed for the first time using microarray techniques. Specific goals were to determine the pervasiveness of any changes and to uncover potential mechanisms that could explain the metabolic hypoactivity and loss of neural plasticity seen in the retrosplenial cortex (van Groen *et al.*, 1993; Garden *et al.*, 2006). The lack of any evidence of neuronal-cell loss in the retrosplenial cortex following ipsilateral thalamic lesions confirmed the appropriateness of the microarray approach and the potential for ‘covert’ pathology. In addition to the biological replications, the microarray results were validated for specific genes using three other techniques (QPCR, *in situ* hybridization and immunohistochemistry), which all revealed very high concordance.

A general point should be made before considering the actual findings. Any disconnection of a brain site will presumably induce some mRNA changes in the target region. It was deliberately decided, however, not to try and contrast the retrosplenial cortex findings with microarray results from another region as each input will have unique properties, as will the target site itself (Vogt *et al.*, 1981; van Groen *et al.*, 1993). As a consequence, there is no natural ‘baseline’ comparison or control condition. For these reasons, the uniqueness of the reported retrosplenial mRNA changes cannot be ascertained without making multiple comparisons with numerous sites, but this is not practicable at present. At the same time it is known that the loss of other afferents to retrosplenial cortex (e.g. from the entorhinal cortex, postrhinal cortex, and laterodorsal thalamic nucleus) do not have marked effects on the two IEGs *c-fos* and *zif268*, strongly indicating that there is a unique relationship between the retrosplenial cortex and the anterior thalamic nuclei. The significance of the present study relates both to a) how the findings inform specific retrosplenial changes already linked to the distal effects of anterior thalamic lesions (e.g. whether any IEGs, in addition to *c-Fos* and *Zif268*, are affected by anterior thalamic lesions and how mRNA changes might help to explain the loss of LTD in retrosplenial cortex), and b) help to explain why retrosplenial dysfunction (typically hypoactivity) is observed in a wide array of neurological disorders.

As predicted (Jenkins *et al.*, 2004), the IEG *c-fos* was hypoactive on the ‘Lesion’ side. This hypoactivity extended to other transcription factors (e.g. *brd8*, *fra-2*, *klf5*, *nfat5*, *neuroD1*, *nfya*, *nr4a1*, *RXRγ*, *smad3*, *smarcc2*, and *zfp91*), so providing a fuller picture of how pervasive the changes in cellular function might prove. Markers for energy metabolism were also strikingly affected, another key indicator of the functional status of the retrosplenial cortex cells after anterior thalamic lesions. Energy metabolism and synaptic activity are inter-related (Williams *et al.*, 1998; Vaynman *et al.*, 2006). Glutamate metabolism depends on astrocyte function (Hertz and Zielke, 2004), while synaptic plasticity and energy metabolism are coupled through neuron-astroglia interactions (Hyder *et al.*, 2006). Numerous pathways involved in these exchanges appear to be affected by the lesion, as depicted in Fig. 5.

Cell signaling appears to be grossly affected by the surgical treatment. The results of the current study suggest the possibility that anterior thalamic nuclei lesions result in the promotion of some glutamatergic-related activity in Rgb. This change could be a combined result of the loss of glutamatergic inputs and the disinhibition of GABA regulation within the cortex. The latter may be mediated via the GABA(A) receptor delta subunit, important for tonic inhibition (Mody *et al.*, 2001; Petrini *et al.*, 2004), and for which the gene transcript *gabrd* was reduced in the lesioned hemisphere in both the microarray and the *in situ* hybridization studies.

GABAergic, serotonergic, adrenergic as well as potassium, sodium and calcium channel activity all appear to have been affected by the thalamic lesions. These effects are likely to produce functional changes in neuronal excitability and firing pattern modulation, and this is reflected in recent work (Garden *et al.*, 2006) that evaluated the effect of anterior thalamic nuclei lesions on single unit recordings in retrosplenial cortex slices. Recordings were taken in retrosplenial cortex layer II neurons after stimulation in layer II or V. In unilateral lesion preparations, LTD could be induced in layers II and V of the 'Intact' cortex but not in layer II in the 'Lesion' hemisphere (Garden *et al.*, 2006). This selective lack of plasticity in the superficial layers in the retrosplenial cortex after anterior thalamic lesions accords both with the only mRNA data looking at different cortical lamina i.e. the changes in *gabrd* transcripts (Exp. 2), and with the previously described superficial reductions of c-Fos and Zif268 proteins after lesions of the anterior thalamic nuclei (Jenkins *et al.*, 2004).

The dendrites of the NMDA-dependent layer II-III Rgb interneurons (Li *et al.*, 2002) are physically associated with those of cells receiving anterior thalamic projections (Ichinohe and Rockland, 2002). The anterior thalamic lesion may result in disruption of the Rgb intrinsic and extrinsic inhibitory/excitatory balance. Like interneuron activity, clustering of delta-subunit containing GABA(A) receptors regulates excitatory signal integration (Mody *et al.*, 2001; Petrini *et al.*, 2004). Together, these changes may reflect altered cortical feed-forward inhibition, producing deficient excitatory input summation and poor spike generation (Daw *et al.*, 2007), contributing to the observed Rgb energy hypometabolism, in addition to potentially deficient energy functions (cf. Fig. 5 for inter-related energy and neuronal activity functions).

The apparent reduction in retrosplenial cortex mitochondrial activity is of potential relevance to Alzheimer's disease. In the earliest stages of Alzheimer's disease, the anterior dorsal nucleus of the thalamus and the entorhinal cortex are among the few regions displaying structural changes (Braak and Braak, 1991b). These regions may be preceded by transentorhinal pathology, which coincides with a "clinically silent" phase, according to the staging by Braak and Braak (1991b). Both the entorhinal cortex and the anterodorsal thalamic nucleus are connected to the retrosplenial cortex, the latter with particularly dense connections (Insausti *et al.*, 1997; van Groen and Wyss, 2003). Furthermore, the anterior thalamic nuclei are one of the few regions that exhibit hypoperfusion in patients that convert to Alzheimer's disease (Johnson *et al.*, 1998).

Interestingly, the progression of Alzheimer's disease exhibits a discrepancy in retrosplenial cortex between the time-course of overt pathology and metabolic disturbance (e.g. Matsuda *et al.*, 2002). While the retrosplenial area does not typically exhibit overt pathology in early Alzheimer's disease, the same region displays hypometabolism from the earliest stages of the disease. The severity of the memory loss is correlated with posterior cingulate hypometabolism but not with medial temporal lobe hypometabolism (Salmon *et al.*, 2000; Matsuda *et al.*, 2002).

The memory loss in Alzheimer's disease is, however, correlated with atrophy in the medial temporal lobe but not in the posterior cingulate cortex (Baron *et al.*, 2001; Chételat *et al.*, 2002; Chételat *et al.*, 2003). Clinical studies, as well as animal experiments, have led to the suggestion that hypometabolism in adults with dementia of the Alzheimer type may be the result of neuroanatomical disconnection (e.g. Fazio *et al.*, 1992; Meguro *et al.*, 1999; Aupée *et al.*, 2001). Studies into the importance of the anterior thalamic nuclei for human memory (Aggleton and Brown, 1999) and studies into the impact of selective anterior thalamic lesions in rodents (Jenkins *et al.*, 2004) not only provide support for this disconnection explanation but also point to the key role of the anterior thalamic nuclei. The present findings add considerable weight to this view as they reveal potential mechanisms underlying some of these effects.

There is some evidence that, just as hypometabolism precedes detectable structural changes in Alzheimer's disease, so does a reduction in synaptic efficacy (Ho *et al.*, 2001; Small *et al.*, 2001; Selkoe *et al.*, 2002; Yao, 2003). Interestingly, the effect in rats of anterior thalamic lesions on retrosplenial cortex IEG activation, which occurs soon after surgery, is both long-lasting and constant (from 1 week to ~10 months). The loss of IEGs is especially dramatic in the superficial laminae of the retrosplenial cortex (Jenkins *et al.*, 2004; Poirier *et al.*, 2005). This is of particular relevance as in Alzheimer's disease the superficial layers of posterior cingulate cortex exhibit severely reduced levels of cytochrome oxidase (Valla *et al.*, 2001). Furthermore, beta-amyloid infusions into the anterior thalamic nuclei produce Rgb GABA reductions (Gonzalo-Ruiz, 1999). This final result is notable given the *gabrd* reductions found in the present study after the selective thalamic lesions.

Several other genes for which expression patterns appear to have been altered and which may be associated with the retrosplenial cortex dysfunction have known associations with processes related to Alzheimer's disease. These include *mmp9*, *chgB*, and *Hsd11b1* (Marcus *et al.* 1998; Lorenzl *et al.* 2003; Marksteiner *et al.* 2000; de Quervain *et al.* 2004). Some of the transcripts found to be differentially expressed are presenilin ligands (e.g. *ubqln1*, *NCS-1*), ApoE ligands (e.g. *LRP3*), or ApoB processors (*APOBEC1*). Alterations were also found in transcripts having known associations with Lewy bodies (*ubqln1*, *neft*). Metals have been found to be differentially regulated in neurodegenerative disorders (Roloff and Platt, 1999), and *pank4*, lower in the 'Lesion' hemisphere, is an isoform of pantothenate kinases, which play a role in iron deposition and neurodegeneration (Thomas and Jankovic, 2004). Meanwhile, levels of the metal selenium were reported to be reduced in the temporal lobe of persons with Alzheimer's disease (Wenstrup *et al.*, 1990), and the *selenoprotein M* (*selm*) gene has also been found to be expressed at lower levels in mice over-expressing human presenilin-2 in comparison to their wild-type littermates (Hwang *et al.*, 2005). *selm* was lower in the 'Lesion' hemisphere in the current study, and selenium deficits can augment the effects of glutamate overactivity (Savaskan *et al.*, 2003).

In summary, tissue that appears normal when examined with standard histological methods reveals evidence of widespread alterations, including hypoactivity, even though it is distal to the site of overt pathology. These findings mirror reports of posterior cingulate hypometabolism following thalamic atrophy in amnesic patients, again suggestive of alterations in regions distal to those exhibiting overt pathology (e.g. Fazio *et al.*, 1992; Reed *et al.*, 1999; Chételat *et al.*, 2003). Our results also address the controversy over the existence and functional relevance of "covert" pathology (Bachevalier and Meunier, 1996; Squire and Zola, 1996). The widespread transcriptional abnormalities lend further support for the notion that the retrosplenial cortex is responsible for cognitive deficits even though its apparently intact appearance has led it to be largely ignored in these conditions. The present study provides candidate genes and, thereby, mechanisms involved in the retrosplenial dysregulation, and hence a better characterization of the susceptibility of the

retrosplenial cortex to distal damage. These results further our understanding of the effects of insults to the brain and reinforce the view that we increasingly need to consider the contributions from regions without overt pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a) Timetable on experimental day. Animals were individually placed for 20 minutes in a novel cage in a novel environment, then returned to the test room (either 10 min, 1h40min or 7h40min). b) Approximate location of the coronal slices along the rostro-caudal axis, and c) the location of the tissue punches on a coronal plane (adapted from Paxinos and Watson, 1997). ATN, anterior thalamic nuclei; Rgb, granular b retrosplenial cortex; IHC, slices taken for Nissl stains and immunohistochemistry.

Figure 2.

a) The smallest and largest anterior thalamic nuclei lesions are represented in dark and light grey, respectively, on a series of standard coronal sections. The numbers refer to the distance from bregma. b) Photomicrograph of a Nissl-stained coronal section, contrasting the anterior thalamic nuclei in a normal hemisphere (left) and a lesioned hemisphere (right). The outline highlights the area of gliosis and cell loss. Note that the tissue was not perfused, hence the poor differentiation. AD, anterior dorsal thalamic nucleus; AM, anterior medial thalamic nucleus; AV anterior ventral thalamic nucleus. The scale bar represents 500 um.

Figure 3.

a) Photomicrographs of representative sections showing the very clear c-Fos but not Nissl, reduction indicated by the arrow in Rgb ipsilateral to the anterior thalamic nuclei lesions. The laminae are identified in each photomicrograph. The scale bar represents 200 μm . b) The selective loss of c-Fos protein is shown by the normal pattern of signal intensity in the cresyl violet sections (upper) compared with the c-Fos labelled sections (lower), with 0-600 μm corresponding to laminae I-VI. Note the difference in the superficial laminae. The horizontal mean profile was derived by analySIS^{AD} (Olympus, UK). Measures of the grey value intensity of each pixel were made along a horizontal plane, and all of these were averaged to produce a laminar profile of signal intensity. (c) Significant reduction of c-Fos-positive cells but not Nissl-stained cells in the Lesion hemisphere (** $p < 0.0001$, see text).

Figure 4.

Hierarchical pathway prediction for the Rgb de-regulation associated with the c-Fos production deficit. Representative microarray-derived Rgb genes were annotated into different functional classes, and grouped according to either molecular function (e.g. transcription factor c-Fos) or biological process (e.g. neurotransmission molecule Gabrd). This hierarchical organization of groups of genes affected by the lesion provides the basis for pathway predictions. Representative genes only are provided here in order to maintain clarity.

Left pathway: The transcription factor Fra-2 is phosphorylated and regulates expression of the RGS4 gene (D.A. Carter, unpublished work). RGS4 affects neurotransmission (via GTPase activation). Altered neurotransmission is manifested in altered LTD/P.

Right pathway: The transcription factor c-Fos is phosphorylated and can be sorted to the mitochondrial genome where it associates with mitochondrial DNA (Ogita *et al*, 2002). Altered mitochondrial function can affect synaptic plasticity (cf. text).

Figure 5.

Schematic depiction of some pathways disrupted by the lesion, involving oxidative phosphorylation (all mitochondrial complexes I-V), the tricarboxylic acid cycle (TCA, Krebs cycle), glutamate-glutamine cycling, and glucose uptake and metabolism. Genes found altered in the current study and associated with these pathways are identified in red. Glu, glutamate; Gln, glutamine.

Figure 6.

QPCR confirmation of candidate genes selected from microarray results. The candidates were selected from the sets of genes derived from fold-change microarray analysis. (S = 'Intact'; L = 'Lesion').

Figure 7.

a) The smallest and largest lesions are represented in dark and light grey, respectively. The plates, from left to right, represent the distances -0.92 , -1.4 , -1.8 , -2.12 , and -2.56 mm from bregma. b) Photomicrograph of a Nissl-stained coronal section, contrasting the anterior thalamic nuclei in a normal hemisphere (left) and a lesioned hemisphere (right). The outline highlights the area of gliosis and cell loss. Note that the tissue was not perfused, hence the poor differentiation. AD, anterior dorsal thalamic nucleus; AM, anterior medial thalamic nucleus; AV anterior ventral thalamic nucleus. The scale bar represents 500 μm . c) Anterior thalamic lesions reduced Rgb *gabrd* expression in a superficial (II) but not a deep (V) lamina. Error bars represent the standard error of the mean, but note that the comparisons are within-subject. $*p < 0.05$. d) Photomicrographs (100 $\times$) of small dark silver grains associated with superficial Rgb laminae II in an 'Intact' (left) and 'Lesion' (right) hemisphere. The scale bar represents 10 μm .

Table 1
Sets of genes exhibiting similar expression patterns as determined by cluster analysis (based on fold-change list).

Set	Found in class patterning? $\ddagger$	Time point (h)			Functional classification	Common Name	Synonym	EST [*]	Genbank
		0.5	2	8					
1	y	✓	-	-	Ion transport	Sodium channel, voltage-gated, type I, beta polypeptide (Scn1b)	GEFSP1	-	AF182949
	n	-	-	-	Cell adhesion	Nel-like 1 (Nell1)	IDH3GL, NRP1	-	NM_031069
	y	✓	-	-	Transcription	Kruppel-like factor 5 (intestinal) (Klf5)	IKLF, BTEB-2	-	NM_053394
	y	-	-	-	Development	Netrin-G1a (Ntng1a)	Laminet1	1067 0.0	BM391312
	n	✓	-	✓	Development	Cerebellin 1 precursor protein (cbn1)	Precerebellin-1 gene	908 0.0	A1227829
	n	-	-	✓	Signal transduction	Small inducible cytokine subfamily 8 (cys-xcys) member 14 (Scyb14)	SCYB14, KSL, Kec. BMAC, BRAK, NIAC, MIP-29, bolekine	178 6e-42	BG380414
	y	-	-	-	Folding, sorting, and degradation	Matrix metalloproteinase 9 (MMP9)	Gelatinase b, Macrophage gelatinase, ype V collagenase, 92kD type IV collagenase, CLG4B	-	NM_031055

Set	Found in class patterning? $\ddagger$	Time point (h)			Functional classification	Common Name	Synonym	EST*	Genbank
		0.5	2	8					
2	n	✓	-	-	Cell communication	Chromogranin B (Chgb)	Parathyroid secretory protein; secretogranin I	-	NM_012526
	n	-	-	✓	Cell adhesion	Olfactomedin 3 (olfm3)	Optimedin form B, NOE3	-	AF442822
	y	✓	-	-	Cell adhesion	Limbic system associated membrane protein (Lsmp)	LAMP-1, CD107a, LGP120	-	U31554
	y	✓	-	-	Development	Fibronectin type III domain containing 5 (Fnkc5)	Peroxisomal protein (pepgene, Pep), Pxp, FRCP2, LOC260327	599 4e-168	AI172165
$S > L$	y	✓	-	-	Neuroactive ligand-receptor interaction	5-HT2C receptor (5-HT2cR)	Htr1c	509 3e-141	BF285539
	y	-	-	-	Unknown	BAC clone RP23-312H15 from 16	LOC360483 mRNA	-	AI412090
	y	✓	-	✓	Transcription	Retinoblastoma binding protein 5 (RBBP-5)	RBO 3	396 3e-107	AA946518

Set	Found in class patterning? $\ddagger$	Time point (h)			Functional classification	Common Name	Synonym	EST*	Genbank
		0.5	2	8
y	✓	-	-	Neuroactive ligand-receptor interaction	Adrenergic receptor, beta 3 (Adrb3)		-	NM_013108
	n	-	-	-	Immune system	Cysteinyl leukotriene receptor 1 (Cyslt1, Cysltr1)		-	NM_053641
	y	✓	-	-	Cell communication	Zyxin (zyx)	HED-2	-	AA943537
Unc	n	-	-	-	Transcription	zinc finger protein 644 (Zfp644)		1823e-43	AW916491
	n	-	-	-	Transcription	c-fos		-	BF415939
	n	-	-	-	Lipid metabolism	Hydroxysteroid dehydrogenase, 11 beta type 1 (Hsd11b1)	Corticosteroid 11-beta-dehydrogenase isozyme 1	-	NM_017080
	n	-	-	-	Cell adhesion	Non-processed neuexin I-alpha (Nrnx1)	Nrxn1b	-	NM_021767
	n	-	-	-	Signal transduction	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 1 (Slc9a3r1)	Ezrin-radixin-moesin-binding phosphoprotein	-	NM_021594
	n	-	-	-	Transcription	Zinc finger protein 91 (PZF)	ZFP91	-	BE112093
	y	-	✓	-	Lipid metabolism	hypothetical protein MGC11324, Hypothetical phospholipid and glycerol acyltransferase		3989e-108	AI599365
	n	-	-	-	Transcription	Fos-related antigen, exon 4 (Fra2)		3079e-81	AI031032
	y	-	-	-	Translation	Speckle-type POZ protein (Spop)	TEF2	4762e-131	BF283504

Set	Found in class patterning? [†]	Time point (h)			Functional classification	Common Name	Synonym	EST [*]	Genbank
		0.5	2	8					
S>L	y	-	-	-	Folding, sorting, and degradation	Ubiquitin specific protease 3 (Usp3)		-	AI411205
L>S	y	✓	✓	✓	Immune system	CD74	INVG34, LN2	-	NM_031069
L>S	n	-	-	-	Cytoskeleton, Microtubule and actin- movement related	Desmuslin (Dmn)	KIAA0353, synemin	-	BG373779
L>S	n	-	-	-	Immune system	Protein S alpha (ProS1)	PSA, PS21, PS22, PS23, PS24, PS25	-	U06230
S>L	n	✓	-	-	Cytoskeleton, Microtubule and actin- movement related	Cytoskeleton-associated protein 4 (Ckap4)	CLIMP-63, P63, MGC99554	-	B1278813
L>S	y	✓	✓	✓	Immune response	Rat MHC class II RT1.u-D-alpha chain mRNA, 3' end	HLA-DRA	-	Y00480

[†] Refers to gene, not necessarily the same transcript.

[‡] Level of time condition for which surgical treatment resulted in significant differences in gene expression of 1.8 fold change.

* For Expressed Sequence Tag (EST) similarity searches, the similarity score and the E-value are provided. (S = 'Intact' = solid line; L = Lesion = dashed line).