Anterior thalamic lesions produce chronic and profuse transcriptional deregulation in retrosplennal cortex: a model of retrosplennial hypoactivity and covert pathology

G.L. POIRIER1,5, K.L. SHIRES1,2,3,4,5, D. SUGDEN3, E. AMIN3, K.L. THOMAS2, D.A. CARTER5, AND J.P. AGGLETON1

1School of Psychology, 2School of Biosciences, Cardiff University, Cardiff, UK, 3Division of Reproduction and Endocrinology, School of Biomedical and Health Sciences, King’s College, London, UK, 4Present address: Centre for Cognitive and Neural Systems, Dept of Neuroscience, University of Edinburgh, UK and 5These authors contributed equally to this work

Anterior thalamic lesions are thought to produce ‘covert pathology’ in retrosplennal cortex, but the causes are unknown. Using microarray analyses we tested the hypothesis that thalamic damage causes a chronic, hypofunction of metabolic and plasticity-related pathways (Experiment 1). Rats with unilateral, anterior thalamic lesions were exposed to a novel environment for 20 min, and granular retrosplennal tissue sampled from both hemispheres 30 min, 2 hours and 8 hours later. Complementary statistical approaches (analyses of variance, predictive patterning and gene set-enrichment analysis) revealed pervasive gene expression differences between retrosplennal cortex ipsilateral to the thalamic lesion and contralateral to the lesion. Selected gene differences were validated by QPCR, immunohistochemistry (Experiment 1) and in situ hybridization (Experiment 2). Following thalamic lesions, the retrosplennal cortex undergoes profuse cellular transcriptome changes including lower relative levels of specific mRNAs that are involved in energy metabolism and neuronal plasticity. These changes in functional gene expression might be driven largely by decreases in the expression of genes encoding transcription factors including brd8, c-fos, fra-2, klf5, nfx1, nrf4a1, sma3, smarcc2 and zfp9, with far fewer (nfat5, neuroD1 and RXRγ) showing increases. These findings have implications for conditions such as diencephalic amnesia and Alzheimer’s disease in which both anterior thalamic pathology and retrosplennal cortex hypometabolism are prominent.

Keywords: Alzheimer’s disease, gene, microarray, pathology

INTRODUCTION

There is a growing list of disorders that exhibit memory impairments associated with dysfunction of the retrosplennal cortex. Such disorders include Alzheimer’s disease (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), hypothyroidism (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 disorders marked hypoactivity of the retrosplennal cortex is apparent from reductions in glucose utilization, regional cerebral blood flow and 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 retrosplennal cortex is sufficient to impair retrosplennal function and, thereby, disrupt memory processes.

The anterior thalamic nuclei (ATN) project densely on the retrosplennal cortex (Vogt et al., 1981; van Groen et al., 1993) and there is evidence that these two regions are functionally interdependent (Sutherland and Hoesing, 1993). Furthermore, pathology in the ATN is associated closely 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; Braak and Braak, 1991b) whereas the retrosplennal 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; Nestor et al., 2003b). Such findings indicate that anterior thalamic damage might severely disrupt retrosplennal cortex function, so linking these two regions in a shared set of neurological disorders, all of which display memory loss.

Additional 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 retrosplennal cortex (Rgb). However, the thalamic lesions in that study were 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 Rgb, including a 90% reduction of C-Fos in superficial Rgb (Jenkins et al., 2004). These changes were striking because (1) they represented the greatest loss of IEG products in any brain area, (2) they occurred even though many other afferents to Rgb remained intact, (3) they are selective because 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 (Albasser et al., 2007; Jenkins et al., 2004; Poirier and Aggleton, 2006). Perhaps more remarkable is the finding from a preliminary in vitro study that prior, unilateral lesions of the ATN block the induction of long-term depression (LTD) in slices of ipsilateral Rgb (Garden et al., 2006). This loss of plasticity is confined to the superficial cortical laminae and, hence, in register with the IEG changes, but the microcircuits are intact in this preparation so the abnormality is not 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 pathology is not detected by standard histological means) because there is no overt pathology in the retrosplenial cortex following ATN lesions (van Groen et al., 1993; Jenkins et al., 2004) and the IEG hypoactivity seems to be permanent (Jenkins et al., 2004). These recent findings indicate that the impact of anterior thalamic damage might be amplified via covert pathology in the retrosplenial cortex. In this study, lesions were placed unilaterally in the ATN to compare retrosplenial cortices in the ‘Intact’ and ‘Lesioned’ hemispheres in a within-subjects design that helped to restrict variance.

OBJECTIVES

In the present study we sought to identify candidate molecular mechanisms that might result in 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 cause retrosplenial cortex hypometabolism and disrupt plasticity-associated functions. In Experiment 1, selected gene differences identified by microarray were validated using quantitative polymerase chain reaction (QPCR) and immunohistochemistry. In Experiment 2, in situ hybridization was used to validate used to validate one further gene, gabrd, which was selected because GABA(A) activity is crucial for the regulation of 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 (Experiment 1), and findings verified for particular genes by the use of immunohistochemistry (IHC, Experiment 1), QPCR (Experiment 1) and in situ hybridization (Experiment 2). All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and associated guidelines.

Experiment 1

subjects

Male pigmented rats (Dark Agouti strain; Harlan) 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 ATN because the projections to the retrosplenial cortex remain ipsilateral, which makes it possible to make 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, 75 mg kg\(^{-1}\)), and then placed in a stereotaxic frame (David Kopf Instruments). A craniotomy was made over both hemispheres. Excitotoxic lesions were produced by injecting 0.19 μl of 0.12 M N-methyl-D-aspartate (NMDA; Sigma) made up in phosphate buffered saline (PBS), pH 7.2, into two sites in the same hemisphere using a 1 μl syringe (Hamilton). 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) was subsequently applied topically and all rats also received a subcutaneous injection (5 ml) of glucose saline. Paracetamol was dissolved in the drinking water and rats were observed daily until recovery. A period of six to nine weeks preceded tissue sampling.

Behavioral procedures

Five days before tissue extraction, the animals were housed individually. In order to minimize unwanted disruption the rats were first habituated daily to a separate holding room in which there were no other rats. At 24 hours before 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 min 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 times, 30 min, 2 hours and 8 hours after the onset of exposure to the novel environment (Fig. 1a), each at comparable times of day. The three delays allowed changes to be detected 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 (Rg). 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, Rg was the region of interest for three principal reasons. First, Rg receives dense inputs from the ATN (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). Third, 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 17–20 hours 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 (8 hours, 2 hours and 0.5 hours prior to sacrifice) (Fig. 1a). Following removal from the skull, the brain was rinsed in cold, DEPC-treated 0.1 M PBS, and placed dorsal-surface up in a Perspex rat brain matrix (WPI; specific for rats weighing 175–300 g). The dissection lasted, at most, 2 min. Two, 2 mm coronal slices were first obtained by making three coronal cuts (Fig. 1b), and then placed on dry ice. Discrete samples of Rg 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 and 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 six 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 ATN lesions**

The lesions were confirmed in two ways. First, coronal sections from the IHC (Fig. 1b) were Nissl stained (the ATN lie just rostral to the most anterior slice taken for Rg tissue). Second, coronal sections from the most rostral portion of Rg (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 Rg (Jenkins et al., 2004).

The portion of the rat brain anterior to the most rostral coronal slice (Fig. 1c) was placed overnight in 4% paraformaldehyde in PBS for fixation and then again overnight in 25% sucrose in PBS. Coronal sections (40 μm) were cut 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 mounted on 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 10 min, 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 10 min 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 then rinsed for 20 min in PBST, four times. 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 analySIS®D program (Olympus). The threshold was set at the same level for all sections and counts made in a frame area (1768 × 1331 µm, using 5× magnification) that included all Rgb lamina. Typically, counts were taken from three consecutive sections from both hemispheres of the retrosplenial cortex (approximately between −2.56 and −3.14 from bregma (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 (Chomczynski and Sacchi, 1987) from the pooled tissue (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 min incubation on ice, samples were centrifuged for 10 min at 14,000 rpm 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 1 hour for the RNA to precipitate. This solution was centrifuged again for 10 min and the resulting pellet dissolved in the denaturing solution and then precipitated with ethanol at −20°C for 1 hour. After a further 10-min centrifugation, the pellet was washed in 95% ethanol, dried and dissolved in diethyl for 1 hour. After a further 10-min centrifugation, the pellet turing solution and then precipitated with ethanol at −20°C for 1 hour. Then sodium acetate, phenol and chloroform-iso-amyl alcohol mixture were added and the solution shaken vigorously. After 10 min incubation on ice, samples were centrifuged for 10 min at 14,000 rpm 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 1 hour for the RNA to precipitate. This solution was centrifuged again for 10 min and the resulting pellet dissolved in the denaturing solution and then precipitated with ethanol at −20°C for 1 hour. After a further 10-min 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 (~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 which 100 ng 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 an Affymetrix 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−1 tRNA. QPCR analysis of transcript levels was performed according to established protocols (Sugden, 2003). Briefly, QPCR was performed using a LightCyclerTM 1.2 (Roche) in a 10 µl volume using sense and anti-sense primers (0.5 µM; sequences which is available as "Supplementary data" on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1472928808000368) and the dNTPs, Taq DNA polymerase and reaction buffer provided in the QuantiTect SYBR Green kit (Qiagen). 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 Tm. 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 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 (r2) > 0.9990 (see QPCR Assay Characteristics which is available as "Supplementary data" on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1472928808000368).

Analyses of microarray data
Analyses used GeneSpring™ software (Version 6.1; Silicon Genetics) 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.

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
Initially we 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 because such data sets exhibit good correlation with normality (Giles and Kipling, 2003). A two-way ANOVA using Surgical Treatment (‘Intact’ and ‘Lesion’) and Time (30 min, 2 hour and 8 hour 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 (FDR) of 0.05 (Benjamini and Hochberg, 1995), which means that 5% of the genes that pass the statistical test are 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 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™. Whereas an ANOVA on differences in gene-expression levels based on fold change might allow the production of a reasonable list of genes that are 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 of 1.8 are biologically meaningful, so it is possible that subtle, but robust, findings might be hidden by such an analysis (Murphy, 2002; Pavlidiis, 2003).

The class prediction function of GeneSpring™ allows the statistical comparison of gene expression levels between treatments, without any arbitrary fold-change threshold. Only 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 predicts treatment in any condition of either duplicate.

The class prediction function uses Fisher’s exact test to assign a P-value for the possibility that transcripts are 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 2%. 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 because 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.mcw.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/).

Analysis of transcription factor binding sites: 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. Similar to the treatment profiling approach described earlier, GSEA compared the two surgical conditions. However, rather than being based on analyses of individual genes, the strength of GSEA lies in the evaluation of the over-representation of predefined gene sets that are pathway-related (Subramanian et al., 2005). This approach enabled 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
GABA-mediated 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 (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) weighing 200–225 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 habituated extensively 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 Experiment 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, frozen on dry ice and 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)-coated glass slides (0.02 mg ml⁻¹ in 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 hybridization**

A DNA antisense probe complementary to nucleotides 119–159 of the rat *gabrd* gene (NCBI accession number NM_017289) (Shivers *et al.*, 1989) was synthesized commercially (Sigma-Genosys). This oligonucleotide was 3' end-labelled with [α-35S]dATP (1200 Ci mmol⁻¹; New England Nuclear) in a 30:1 molar ratio of radiolabeled 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 × 10⁶ d.p.m. ml⁻¹. 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 hybridization, sections were opposed to BioMax X-ray film (Eastman Kodak) for two weeks. After obtaining appropriate x-ray film exposures, sections were dipped in K5 photographic emulsion (Ilford). Sections were exposed for 18 weeks at 4°C before developing 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 in the analyses exhibited discrete lesions in the ATN, removing most of the cells in the anterodorsal and anteroventral nuclei (Fig. 2). In the smallest of these 12 lesions there was light sparing of the anterior ventral nucleus throughout, although 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 in either the anterior medial nucleus or the lateral dorsal nucleus. 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

---

Fig. 2. (A) The smallest and largest ATN 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 ATN in a normal hemisphere (left) and a lesioned hemisphere (right). The outline highlights the area of gliosis and cell loss. The tissue was not perfused, hence the poor differentiation. Abbreviations: AD, anterior dorsal thalamic nucleus; AM, anterior medial thalamic nucleus; AV, anterior ventral thalamic nucleus. Scale bar, 500 μm.
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, thus, no set was removed (Fig. S1, which is available as “Supplementary data” on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1472928808000368). All samples were also correlated highly (>0.90, 0.25 weighing coefficient). The data quality filters yielded 9075 satisfactory ’transcripts’ out of the original 15923 probe sets. All comparisons are between the ‘Intact’ and the ‘Lesion’ hemisphere, so the transcript analyses are based on relative, 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 fold-change in gene expression identified 32 genes with significant changes in expression levels between the surgical conditions (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, these 32 genes were expressed at a relatively higher level on the ‘Intact’ than the ‘Lesion’ side (25 compared to 7 genes, respectively). Among the genes with a 1.8-fold change in expression were several transcription factors (c-fos, klf5, fra-2 and zfp91), which have functional associations with cellular processes and genetic information processing, most prominently relating to immune responses and the regulation of transcription.

Although there was no overall effect of post-novelty time interval on gene expression, cluster analysis on this list identified sets of genes with three major temporal patterns of expression according to condition (Table 1). One-way ANOVA identified the times at which there was a 1.8-fold change in expression of each gene between the two surgical treatment conditions (Table 1). The complementary nature of the profiles of these cluster analyses helps to explain why there was an overall null effect of post-novelty interval. Furthermore, although there were no systematic differences in the post-lesion durations for the three novelty context intervals, variability in this measure might increase the likelihood of a null result.

Treatment transcriptome profiling
Class prediction analysis identified 202 genes (Table 2, which is available as “Supplementary data” on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1472928808000368) for which the 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). Expression of >75% of the 202 genes identified by this method was higher in the ‘Intact’ than ‘Lesion’ Rgb tissue (157 compared to 45, respectively). Cluster analysis again allowed us to further segregate the genes identified into sets according to their expression patterns across the time points for each condition (Table 2, which is available as “Supplementary data” on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1472928808000368).

Both the fold change and the predictive patterning analyses specified that expression of genes that encode proteins in the nucleus and the membrane appeared to be affected more than those associated with other cellular compartments. Furthermore, transcription was one of the functions represented most prominently in the expression patterns; hence, the complementary nature of the profiles of these cluster analyses helps to explain why there was an overall null effect of post-novelty interval.

Fig. 3. (A) Photomicrographs of representative sections showing reduction of c-Fos but not of Nissl, indicated by the arrow in Rgb ipsilateral to the ATN lesions. The laminae are identified in each photomicrograph. Scale bar, 200μm. (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 0–600μm corresponding to laminae I–VI. Note the difference in the superficial laminae. The horizontal mean profile was derived by analySIS®D (Olympus). 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).
Table 1. Sets of genes with similar expression patterns, determined by cluster analysis (based on fold-change list).

<table>
<thead>
<tr>
<th>Set</th>
<th>Found in class patterning?</th>
<th>Time point (h)</th>
<th>Functional classification</th>
<th>Common Name</th>
<th>Synonym</th>
<th>EST*</th>
<th>Genbank</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 S &gt; L</td>
<td>y</td>
<td>0.5</td>
<td>Ion transport</td>
<td>Na⁺ channel, voltage-gated, type 1, beta polypeptide (Scn1b)</td>
<td>GEFS1</td>
<td>–</td>
<td>AF182649</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>2</td>
<td>Cell adhesion</td>
<td>Nel-like 1 (Nell1)</td>
<td>IDH1, GL, NRP1</td>
<td>–</td>
<td>NM_023069</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>8</td>
<td>Transcription</td>
<td>Kruppel-like factor 5 (intestinal) (Klf5)</td>
<td>IKLE, BTEB-2</td>
<td>–</td>
<td>NM_053394</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>–</td>
<td>Development</td>
<td>Netrin-G1a (Ntn51a)</td>
<td>Laminet1</td>
<td>1067</td>
<td>BM391312</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>–</td>
<td>Development</td>
<td>Cerebellin 1 precursor protein (clbn1)</td>
<td>Preccerebellin-1 gene</td>
<td>908</td>
<td>AI272849</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>–</td>
<td>Signal transduction</td>
<td>Small inducible cytokine subfamily 8 (cys-cys) member 14 (Scyb14)</td>
<td>SCYB14, KS1, Kec, BMAC, BRAK, NJAC, MIP-29, bolekin</td>
<td>178</td>
<td>BG380414</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>–</td>
<td>Folding, sorting, and degradation</td>
<td>Matrix metalloproteinase 9 (MMP9)</td>
<td>Gelatinase b, Macrophage gelatinase, type V collagenase, 92KD type IV collagenase, CLG4B</td>
<td>–</td>
<td>NM_031055</td>
</tr>
<tr>
<td>2 S &gt; L</td>
<td>n</td>
<td>–</td>
<td>Cell communication</td>
<td>Chromogranin B (Chgb)</td>
<td>Parathyroid secretory protein; secretogranin I</td>
<td>–</td>
<td>NM_012526</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>–</td>
<td>Cell adhesion</td>
<td>Olfactomedin 3 (olfm3)</td>
<td>Optimedin form B, NOE3</td>
<td>–</td>
<td>AF447822</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>–</td>
<td>Cell adhesion</td>
<td>Limbic system associated membrane protein (Lsampl)</td>
<td>LAMP-1, CD107a, LGP120</td>
<td>–</td>
<td>U31554</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>–</td>
<td>Development</td>
<td>Fibronectin type III domain containing 5 (Fndc5)</td>
<td>Peroxisomal protein (pepgene, Pep, FrpC2, LOC260327)</td>
<td>599</td>
<td>4e-168</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>–</td>
<td>Neuroactive ligand-receptor interaction</td>
<td>5-HT2C receptor (5-HT2cR)</td>
<td>Htr1c</td>
<td>509</td>
<td>BF285539</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>–</td>
<td>Unknown</td>
<td>BAC clone RP23-312H15 from 16</td>
<td>LOC560483 mRNA</td>
<td>–</td>
<td>AI412090</td>
</tr>
<tr>
<td></td>
<td>y</td>
<td>–</td>
<td>Transcription</td>
<td>Retinoblastoma binding protein 5 (RBBP-5)</td>
<td>RBQ 3</td>
<td>396</td>
<td>AA946518</td>
</tr>
<tr>
<td>3 L &gt; S</td>
<td>y</td>
<td>–</td>
<td>Neuroactive ligand-receptor interaction</td>
<td>Adrenergic receptor, beta 3 (Adrb3)</td>
<td>–</td>
<td>NM_013108</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>–</td>
<td>Immune system</td>
<td>Cysteinylation leukotriene receptor 1 (Cyslt1, Cyslt1)</td>
<td>–</td>
<td>NM_053641</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>y</td>
<td>–</td>
<td>Cell communication</td>
<td>Zyxin (zyx)</td>
<td>–</td>
<td>AA943537</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>–</td>
<td>Transcription</td>
<td>zinc finger protein 644 (Zfp644)</td>
<td>HED-2</td>
<td>182</td>
<td>AW918491</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>–</td>
<td>Transcription</td>
<td>c-fos</td>
<td>–</td>
<td>BF455939</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>–</td>
<td>Lipid metabolism</td>
<td>Hydroxysteroid dehydrogenase, 11 beta type 1 (Hsd11b1)</td>
<td>Corticosteroid 11-beta-dehydrogenase isozyme 1</td>
<td>–</td>
<td>NM_017080</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>–</td>
<td>Cell adhesion</td>
<td>Non-processed neurexin 1-alpha (Nrxn1)</td>
<td>Nrxn1b</td>
<td>–</td>
<td>NM_021767</td>
</tr>
<tr>
<td>S &gt; L</td>
<td>n</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Signal transduction</td>
<td>Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 1 (Slc9a3r1)</td>
<td>Ezrin-radixin-moesin-binding phosphoprotein</td>
</tr>
<tr>
<td>S &gt; L</td>
<td>n</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Transcription</td>
<td>Zinc finger protein 91 (PZF)</td>
<td>ZFP91</td>
</tr>
<tr>
<td>S &gt; L</td>
<td>y</td>
<td>–</td>
<td>✓</td>
<td>–</td>
<td>Lipid metabolism</td>
<td>Hypothetical protein MGC11234, Hypothetical phospholipid and glycerol acyltransferase</td>
<td>–</td>
</tr>
<tr>
<td>S &gt; L</td>
<td>n</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Transcription</td>
<td>Fos-related antigen, exon 4 (Fra-2)</td>
<td>–</td>
</tr>
<tr>
<td>S &gt; L</td>
<td>y</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Translation</td>
<td>Speckle-type POZ protein (Spop)</td>
<td>TEF2</td>
</tr>
<tr>
<td>S &gt; L</td>
<td>y</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Folding, sorting, and degradation</td>
<td>Ubiquitin specific protease 3 (Usp3)</td>
<td>–</td>
</tr>
<tr>
<td>L &gt; S</td>
<td>y</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>Immune system</td>
<td>CD74</td>
<td>INVG34, LN2</td>
</tr>
<tr>
<td>L &gt; S</td>
<td>n</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Cytoskeleton, Microtubule and actin-movement related</td>
<td>Desmuslin (Dmn)</td>
<td>KIAA0353, synemin</td>
</tr>
<tr>
<td>L &gt; S</td>
<td>n</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Immune system</td>
<td>Protein S alpha (ProS1)</td>
<td>PSA, PS21, PS22, PS23, PS24, PS25</td>
</tr>
<tr>
<td>S &gt; L</td>
<td>n</td>
<td>✓</td>
<td>–</td>
<td>–</td>
<td>Cytoskeleton, Microtubule and actin-movement related</td>
<td>Cytoskeleton-associated protein 4 (Ckap4)</td>
<td>–</td>
</tr>
<tr>
<td>L &gt; S</td>
<td>y</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>Immune response</td>
<td>Rat MHC class II RT1.u-D-alpha chain mRNA, 3’ end</td>
<td>HLA-DRA</td>
</tr>
</tbody>
</table>

*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).
frequently in both the fold-change and the predictive pattern-
ing analyses. In addition to the expected fall in the expression of c-fos, based on previous immunohistochemical studies of c-Fos protein concentration 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 factor genes identified by predictive pattern-
ing included klf5, brdh, nfasf, nfx, 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. Computational analysis of the gene promoter elements identified candidate binding sites for transcription factors that were shared by the genes high-
lighted by the treatment profiling and the fold-change approaches. In descending order, the most common binding sites were for the transcription factors FoxD3, Pax-4, Oct-1 and AP-1 for treatment profiling, and FoxD3, Oct-1 and AP-1 for the fold-change gene lists (Fig. S2, which is available as “Supplementary data” on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1472928808000368). AP-1, but not FoxD3, Pax-4 or Oct-1 was differentially expressed between the surgical conditions. Two of the hetero-
dimeric AP-1 binding partners, c-fos and fra-2, were lowest in the ‘Lesion’ hemisphere in the fold-change list (Table 1). It is, thus, plausible that these two transcription factors have an important role in regulating the altered retrosplenic cortex response.

Both analyses yielded candidates underlying biological function changes, and we have no a priori reason to believe that the results of one type of analysis is more important than the other. Thus, to simplify the description of the data, we integrated the results of the fold-change and the treatment profiling analyses because they indicate many similar altera-
tions in gene expression.

We found evidence of widespread alterations in the expression of genes involved in many biological functions (Fig. 4). These alterations are most likely to be chronic effects because acute recovery processes, such as an increase in c-Fos, are thought to peak 3–10 days after injury and return to baseline before one month (Nieto-Sampedro et al., 1982; Buytart, 2001; Mingorance et al., 2005). In the present study the changes indicated potential differences in neuroendocrine activity (e.g. hsd11b1, RXRγ, brd8 and adcyap1), cell adhesion, growth and reorganization (e.g. cyclinD2, klf5, lasp, neuroD1, nptx2, reprim, actin alpha-1, cbln1, lasp, map2, mnpq, mnpq2, myosin Ie, myosin IXa, nelf, nefh, netrin1, netrin-g1a, neurexin1, neureti-
tin1, plexinb2, sclib 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 and tore). We focus on gene expression changes that reflect energy metabolism and neuroplasticity, because these best address the goals of the experiment.

Energy production
Expression differences were found that indicate disturbances in blood, oxygen and energetic activities (Fig. 5). The ‘Lesion’ hemisphere contained lower levels of transcripts associated with effective hemoglobin oxygen transport (hba1 and urod) and glucose transport and metabolism (exoc7, ehd2 and pank4) (Ishiki and Klip, 2005; Yunfeng et al., 2005). Additionally, the higher expression of COX1/Ptgs1 after the lesion might be associated with an alteration in oxygenation and metabolism because this molecule is an important mediator of neurovascular coupling and vasodilation (Takano et al., 2006). There were pervasive alterations in expression of mitochondrial genes, which have an important role in oxidative phosphorylation and can affect the gluta-
mine–glutamate cycle. Deficient metabolic function is indi-
cated by the finding that mitochondrial transcripts exhibited altered expression patterns in all mitochondrial complexes, and were almost exclusively lower in the ‘Lesion’ condition (ndufy2, ndufa8, ndufb6, CoQ9, UCR/QCR9, cox6a, atp5a, atp6s14, mdh2, sdhc, but not cox6b) (Fig. 5; Table 2, which is available as “Supplementary data” on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1472928808000368). GSEA replicated this finding because gene sets for the electron transport chain (FDR = 0.044) and oxidative phosphorylation (FDR = 0.040 which is available as “Supplementary data” on Cambridge Journals Online: http://www.journals.cup.org/abstract_S1472928808000368) were both relatively enriched in the ‘Intact’ condition. These were the only enriched sets with FDR < 0.05 in that condition.

c-Fos and the AP-1 complex can translocate to the mito-
chondrion, 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 that are altered in the current study identified potential AP-1 binding sites on several mito-
chondrial genes, including ndufy2, ndufa8, COQ9, atp6s14 and mdh2 (all reduced on the lesioned side). Although plausible, that the deficit in c-fos expression might be linked directly to the mitochondrial deficits was not tested in the present study.

Neuroplasticity
Some of the genes that are differentially regulated in this study have a role in neuroplasticity. For example, the activity of some transcripts that were found in lower abundance on the ‘Lesion’ side are associated with either long-term potentiation (LTP), including adcyap1 (Otto et al., 2001; Matsuyama et al., 2003), carbonic anhydrase 4 (White and Platt, 2001), mmp9 (Nagy et al., 2006), nes-1 (Genin et al., 2001; Brackmann et al., 2004) and neureti-
tin1 (Wibrand et al., 2006), ppp1r1a (Allen et al., 2000) or long-term depression (LTD), including cbh1 (Hirai et al., 2005), Nr4a1 (Lindecke et al., 2006), ppp1r1a (Mulkey et al., 1994; Morishita et al., 2001; Jouveneau et al., 2006) and Prkar1b (Brandon et al., 1995). In contrast, in the ‘Lesion’ side there were higher levels of nptx2, which is LTPrelated (Wibrand et al., 2006), and RXRγ, which is 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 more transcripts associated with synaptic signaling (e.g. scamp1, syt5, napa, ap2m1, ncs-1, exoc7, lrp3 and neurexin1, but lower for ehd2). Neuronal signaling from the ATN to the retrosplenic cortex relies on glutamatergic inputs (Gonzalo-Ruiz et al., 1997) and their interaction with GABA-releasing interneurons within the cortex (Ichinohe and Rockland, 2002). It is, thus, not surprising to find evidence of altered regulation of both neurotransmitters.
Whereas no glutamate receptors were found to be differentially regulated, several transcripts indicate a disturbance in glutamate-mediated functions (Fig. 5). An immediate-early effector gene, \textit{nptx2}, which is reported to be involved in selective clustering of glutamate receptors on interneurons (O’Brien et al., 1999; Mi et al., 2002) was higher 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 function were also affected by the ATN lesion, and, likewise, point to upregulation of glutamate-mediated activity. NMDA receptor antagonism leads to increases in glutamine synthetase and also in extended tricarboxylic acid cycling (Brenner et al., 2005). Accordingly, NMDA hyperactivity might be associated with a reduction in glutamine synthetase and a decrease in tricarboxylic acid cycling. In the present experiment, the finding of a lower expression on the ‘Lesion’ side of gene transcripts that encode glutamine synthetase, malate dehydrogenase and succinate dehydrogenase is consistent with reduced activity of the tricarboxylic acid cycle and a potential increase in NMDA-receptor stimulation.

![Diagram of predicted pathways](image)

**Predicted pathways**

Whereas no glutamate receptors were found to be differentially regulated, several transcripts indicate a disturbance in glutamate-mediated functions (Fig. 5). An immediate-early effector gene, \textit{nptx2}, which is reported to be involved in selective clustering of glutamate receptors on interneurons (O’Brien et al., 1999; Mi et al., 2002) was higher 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 function were also affected by the ATN lesion, and, likewise, point to upregulation of glutamate-mediated activity. NMDA receptor antagonism leads to increases in glutamine synthetase and also in extended tricarboxylic acid cycling (Brenner et al., 2005). Accordingly, NMDA hyperactivity might be associated with a reduction in glutamine synthetase and a decrease in tricarboxylic acid cycling. In the present experiment, the finding of a lower expression on the ‘Lesion’ side of gene transcripts that encode glutamine synthetase, malate dehydrogenase and succinate dehydrogenase is consistent with reduced activity of the tricarboxylic acid cycle and a potential increase in NMDA-receptor stimulation.

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

Evidence of perturbations in glutamate function also arises from apparent alterations in kynurenine metabolism. Transcripts encoding cyclooxygenase 1 (COX1/ptgs1) and kynurenine 3-monooxygenase (KMO), enzymes that are associated with the metabolites of this pathway, were relatively higher 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; Erhardt and Engberg, 2002), and quinolinic acid act as antagonist and agonist, respectively, at NMDA receptors. Reduced concentrations of KMO cause kynurenic acid to increase (Carpenedo et al., 2002; Erhardt and Engberg, 2002). COX1 is associated with NMDA activity and modulation of kynurenic acid levels. NMDA activation increases levels of this enzyme (Pepicelli et al., 2005) and reduction in COX1 increases kynurenic acid levels (Schwieler et al., 2005).

Finally, adenylate cyclase activating polypeptide 1 (adcyap1) increases the re-uptake of glutamate by glia (Figiel and Engele, 2000). A lower concentration of adcyap1 on the ‘Lesion’ side might be associated with greater glutamate-mediated activity in this hemisphere. This observation, and the evidence presented above, indicate a disturbance in glutamate-mediated signalling, which is potentially associated with an increase in NMDA activity in Rgb.

Transcripts for other neurotransmitter receptors and enzymes, in addition to several Na+ and K+ ion channels, appear to display robust, consistent differences between the
surgical treatment conditions. Such changes were apparent for GABA-mediated signaling, including gabrd and ubqln1 (a mediator of GABA receptor subunit composition) (Bedford et al., 2001), and also noradrenaline- (adrb3 and adrbk1) and 5-HT-mediated signaling (HT2C). Transcripts encoding HT2C were lower on the ‘Lesion’ side, which is consistent with previous findings for 5HT1B, whereas those for Adrb3 were relatively higher on the ‘Lesion’ side, which is opposite to findings for Adrb2 (van Groen et al., 1993).

QPCR validation of candidate genes
Fig. 6 shows the total number of transcripts in 2 μl, normalized to β-actin, for the mRNA of nine preselected genes. The QPCR results were highly consistent with the microarray results, strengthening confidence in the overall findings of the microarray analyses. All but one of the gene time-point values (see below) revealed relative patterns of expression that were similar to those seen in the microarray analyses (26 out of 27). The 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 gene, gabrd, which was reduced in the ‘Lesion’ hemisphere. This gene was chosen because GABA(A) activity is crucial in regulating glutamatergic afferents to the cortex (Daw et al., 2007).

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

Again, 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 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 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 ATN, extended to many functions, pervading cellular activities represented by the transcriptome.
- Replicating several findings using a different technique, QPCR, validated changes in the transcriptome.
- Important groups of molecules associated with essential functions, including energy and oxygenation, neuroplasticity, and cell signaling from synaptic to intracellular communication, exhibited altered transcription patterns.
- Transcription factors with altered expression levels were not restricted to either c-Fos or AP-1 dimer members. These transcription factors might have an important role in mediating the response of the retrosplenic cortex to ATN lesions. Computational analyses revealed that AP-1 binding sites are among the most common binding sites for transcription factors on the genes affected by the lesion. Via c-fos, the concentration of AP-1 was the most common transcription factor affected by the lesion. By contrast, although FOXD3 has the most binding sites on genes affected by the distal lesion, expression of FOXD3 was not altered.
- The greater sensitivity of the superficial laminae of the retrosplenic cortex to anterior thalamic lesions was reflected in a lamina-specific reduction of a specific GABA receptor subunit.

DISCUSSION

Dysfunction of the retrosplenic cortex following lesions of the ATN was analyzed globally for the first time using microarray techniques. Specific goals were to determine the pervasiveness of any changes and potential mechanisms that might explain the metabolic hypoactivity and loss of neural plasticity seen in the retrosplenic cortex (van Groen et al., 1993; Garden et al., 2006). The lack of evidence of neuronal-cell loss in the retrosplenic cortex following ipsilateral thalamic lesions confirmed that the microarray approach was appropriate, 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 demonstrated concordance.

A general point should be made before considering the findings. Disconnection of a site in the brain will, presumably, induce some changes in mRNA in the target region. However, we decided not to compare findings in the retrosplenic cortex with microarray results from another region because 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 retrosplenic mRNA changes cannot be ascertained without making multiple comparisons with numerous sites, but this is not practical at present. In addition, we know that the loss of other afferents to retrosplenic cortex (e.g. from the entorhinal cortex, postrhinal cortex and laterodorsal thalamic nucleus) have no marked effects on the immediate early genes (IEGs) c-fos and zif268, which strongly indicates that there is a unique relationship between the retrosplenic cortex and the ATN.

The significance of the present study relates both to (a) how the findings inform specific retrosplenic changes that are 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 retrosplenic cortex), and (b) why retrosplenic dysfunction (typically hypoactivity) is observed in diverse neurological disorders.
As predicted (Jenkins et al., 2004), the IEG c-fos was hypoactive on the ‘Lesion’ side. This hypoactivity extended to genes encoding other transcription factors (e.g. brdb8, fra-2, klf5, nfat5, neuroD1, nfpx, nr4a1, RXRγ, smad3, smarcc2 and zfp91), so providing a fuller picture of how pervasive the changes in cellular function might be. Markers for energy metabolism were also affected strongly, providing another key indicator of the functional status of cells in the retrosplenial cortex 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), whereas 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 current results indicate that ATN lesions might result in the promotion of glutamate-related activity in Rgb. This change might be a combined result of the loss of glutamatergic inputs and the disinhibition of GABA regulation within the cortex. The latter might be mediated via the delta subunit of the GABA(A) receptor, which is important for tonic inhibition (Mody et al., 2001; Petriti et al., 2004), and for which the gabrd transcript was reduced in the lesioned hemisphere in both microarray and in situ hybridization studies.

Effects of GABA, 5-HT and noradrenaline as well as K⁺, Na⁺- and Ca²⁺-channel activity all appear to be affected by the thalamic lesions. These effects are likely to produce functional changes in neuronal excitability and firing pattern modulation, which is apparent from recent work (Garden et al., 2006) to evaluate the effect of ATN lesions on single-unit recordings in slices of retrosplenial cortex. Recordings were made from retrosplenial cortex layer II neurons after stimulation in either layer II or layer 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; Experiment 2), and with superficial reductions of c-Fos and Zif268 after lesions of the ATN that have been described previously (Jenkins et al., 2004).

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

The apparent reduction in mitochondrial activity in the retrosplenial cortex is of potential relevance to Alzheimer’s disease. In the earliest stages of this disease, the anterior dorsal nucleus of the thalamus and the entorhinal cortex are two of the few regions that display structural changes (Braak and Braak, 1991b). These regions might be preceded by transentorhinal pathology, which coincides with a ‘clinically silent’ phase (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 (Insauti et al., 1997; van Groen and Wyss, 2003). Furthermore, the ATN are one of the few regions that exhibit hypoperfusion in patients that convert to Alzheimer’s disease (Johnson et al., 1998).

Interestingly, during the progression of Alzheimer’s disease there is a discrepancy in retrosplenial cortex between the time-course of overt pathology and metabolic disturbance (Matsuda et al., 2002). Whereas the retrosplenial area does not usually exhibit overt pathology in early Alzheimer’s disease, it does display hypometabolism. The severity of memory loss correlates with in the posterior cingulate but not the medial temporal lobe (Salmon et al., 2000; Matsuda et al., 2002).

Memory loss in Alzheimer’s disease does, however, correlate 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 and animal experiments have led to the suggestion that hypometabolism in adults with dementia of the Alzheimer type might be the result of neuroanatomical disconnection (Fazio et al., 1992; Meguro et al., 1999; Aupée et al., 2001). Studies into the importance of the ATN for human memory (Aggleton and Brown, 1999) and the impact of selective anterior thalamic lesions in rodents (Jenkins et al., 2004) provide support for this disconnection explanation and point to the key role of the ATN. The present findings add considerable weight to this view because they reveal potential mechanisms that underlie some of these effects.

There is some evidence that, like hypometabolism, a reduction in synaptic efficacy also precedes detectable structural changes in Alzheimer’s disease (Ho et al., 2001; Small et al., 2001; Selkoe, 2002; Yao, 2003). Interestingly, in rats the effect of anterior thalamic lesions on IEG activation in the retrosplenial cortex occurs soon after surgery, and 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 because, 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 ATN reduce GABA in the Rgb (Gonzalo-Ruiz, 1999). The latter result is notable given the reduction in gabrd identified after the selective thalamic lesion in the present study.

Several other genes for which expression patterns appear to be altered and that might be associated with dysfunction of the retrosplenial cortex are associated with processes related to Alzheimer’s disease. These include mmp9, chGB and Hsd11b1 (Marcus et al., 1998; Marksteiner et al., 2000; Lorenzl et al., 2003; de Quervain et al., 2004). Some transcripts that were expressed differentially encode presenilin ligands (e.g. ubqln1 and NCS-1), ApoE ligands (e.g. LRPs) and ApoB processors (APOBEC1). Alterations were also noted in transcripts that are associated with Lewy bodies (ubqln1 and nefh). Metals are differentially regulated in neurodegenerative disorders (Roloff and Platt, 1999), and pank4, which is lower...
in the ‘Lesion’ hemisphere, encodes an isoform of the pan-
proliferative kinase enzymes, which have a role in iron depo-
sition and neurodegeneration (Thomas and Jankovic, 2004). The concentration of selenium is reduced in the temporal lobe of people with Alzheimer’s disease (Wenstrup et al., 1990) and the gene encoding selenoprotein M (selm) is also expressed at lower levels in mice that overexpress human presenilin-2 compared to their wild-type littermates (Hwang et al., 2005). In the current study selm was lower in the ‘Lesion’ hemisphere, 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 hypoxia, even though it is distal to the site of overt pathology. These findings are similar to reports of posterior circulate hypometabolism following thalamic atrophy in amnestic patients, again suggestive of alterations in regions that are distal to those that exhibit overt pathology (Fazio et al., 1992; Reed et al., 1999; Chételat et al., 2003). Our results also address the controversy about the existence and functional relevance of ‘covert’ path-
ology (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 identifies candidate genes, thereby indicating mechanisms involved in retrosplenial dysregulation and, hence, 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 need to consider the contributions from regions without overt pathology.

ACKNOWLEDGEMENTS

We thank Prof. Zafrir I. Bashir and Prof. Malcolm W. Brown for their assistance. This research was funded by the Medical Research Council (UK, G9713086 to J.P.A).

REFERENCES

Aggleton J.P. and Sahgal A. (1993) The contribution of the anterior thal-
amic nuclei to anterograde amnesia. Neupropsychologia 31, 1001 –1019.
Allen P.B., Hvalby O., Jensen V., Errington M., Ramsay M., Chaudhry F.A. et al. (2000) Protein phosphatase-1 regulation in the induction of long-term potentiation: Heterogeneous molecular mechan-
isms. Journal of Neuroscience 20, 3537–3543.
Baron J.C., Chételat G., Desgranges B., PercheY G., Landeau B., de laSayette V. et al. (2001) In vivo mapping of gray matter loss with voxel-
Bedford F.K., Kittler J.T., Muller E., Thomas P., Uren J.M., Merlo D. et al. (2001) GABAA receptor cell surface number and subunit stab-
ility are regulated by the ubiquitin-like protein Plic-1. Nature Neuroscience 4, 908–916.
lation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162, 156–159.


Yunfeng L., Yongsheng C., Lifeng Z., Qiping F., Zhuo L., Yongwei Z. et al. (2005) High glucose upregulates pantothenate kinase 4 (PanK4) and thus affects M2-type pyruvate kinase (Pkm2). Molecular and Cellular Biochemistry 277, 117–125.

Correspondence should be addressed to: Guillaume Poirier School of Psychology Cardiff University 70 Park Place Cardiff, UK, CF10 3AT tel: 44 (0) 29 2087 5301 fax: 44 (0) 29 2087 4858 email: PoirierG@cardiff.ac.uk and Kate Shires Centre for Cognitive and Neural Systems Dept of Neuroscience University of Edinburgh 1 George Square Edinburgh, UK, EH8 9JZ tel: +44 131 650 4571 fax: +44 131 651 1835 email: kshire@staffmail.ed.ac.uk