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Research report

Brief isoflurane anaesthesia affects differential gene expression, gene ontology and gene networks in rat brain

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ABSTRACT

Much is still unknown about the mechanisms of effects of even brief anaesthesia on the brain and previous studies have simply compared differential expression profiles with and without anaesthesia. We hypothesised that network analysis, in addition to the traditional differential gene expression and ontology analysis, would enable identification of the effects of anaesthesia on interactions between genes. Rats (n = 10 per group) were randomised to anaesthesia with isoflurane in oxygen or oxygen only for 15 min, and 6 h later brains were removed. Differential gene expression and gene ontology analysis of microarray data was performed. Standard clustering techniques and principal component analysis with Bayesian rules were used along with social network analysis methods, to quantitatively model and describe the gene networks. Anaesthesia had marked effects on genes in the brain with differential regulation of 416 probe sets by at least 2 fold. Gene ontology analysis showed 23 genes were functionally related to the anaesthesia and of these, 12 were involved with neurotransmitter release, transport and secretion. Gene network analysis revealed much greater connectivity in genes from brains from anaesthetised rats compared to controls. Other importance measures were also altered after anaesthesia; median [range] closeness centrality (shortest path) was lower in anaesthetized animals (0.07 [0–0.30]) than controls (0.39 [0.30–0.53], $p < 0.0001$) and betweenness centrality was higher (53.85 [32.56–70.00]% compared to 5.93 [0–30.65]%, $p < 0.0001$). Simply studying the actions of individual components does not fully describe dynamic and complex systems. Network analysis allows insight into the interactions between genes after anaesthesia and suggests future targets for investigation.

1. Introduction

Despite the use of volatile anaesthesia for over 100 years, there is still much to be identified regarding effects on tissues and cells at the molecular level to explain both anaesthetic effects on the brain and also non-anaesthetic effects such as cognitive impairment, conditioning or immune modulation. Much remains unknown about the mechanisms of anaesthesia or the persistent effects of isoflurane on the brain.

The transcriptome (messenger RNA, mRNA) can vary with external environmental conditions and is more dynamic than the genome which is relatively fixed in an individual. Using microarray technology it is possible to investigate changes in the transcriptome across the entire known transcribed genome of several species including mouse, rat and human in organs, or regions of organs, or blood, including in response to anaesthesia.

A few studies have reported the effect of anaesthesia with volatile agents on the transcriptome in cells or various organs from rats [1–5] and have showed that changes can persist even several days after exposure to anaesthetics [4]. Clearly, changes in the transcriptome itself cannot cause molecular effects, but rather the consequent protein and functional metabolic changes. Most studies of effects of anaesthesia on gene transcripts have compared differential expression profiles in samples from animals exposed or not to anaesthesia and grouped the altered genes according to gene ontology groupings by function [2]. We hypothesised that using a systems biology approach with gene connections identified using Bayesian rules and with further analysis using social network tools [6–8] in addition to the traditional differential gene expression and ontology analysis, we would be able to identify novel gene interactions for further investigation. We therefore undertook network

Abbreviations: GOEAST, gene ontology enrichment analysis software; MAC, minimum alveolar concentration; SNARE, *N*-ethylmaleimide-sensitive factor attachment protein receptor.

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analysis on the brain transcriptome of rats several hours after a brief period of isoflurane anaesthesia and also studied differential gene expression using conventional techniques.

2. Methods

2.1. Animals

All animal studies were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986, following relevant aspects of the ARRIVE guidelines [9]. Groups of 20 week old male Sprague Dawley rats were studied (250–300 g). Animals were conventionally housed, allowed food and water *ad libitum* and kept on a 12 h light-dark cycle. All experiments began mid-morning to control for any diurnal effects. Rats were conditioned to handling and the anaesthetic equipment prior to experimentation. Animals were allocated by random number to either exposure to isoflurane in oxygen at a steady state concentration of 2% (1.4× minimum alveolar concentration, MAC) (n = 10) or oxygen only (n = 10) in a transparent gas tight tank, about 30 cm × 20 cm × 20 cm, into which gas flowed at about a litre/minute. After 15 min were placed on a heating mat and allowed to recover and were then returned to their cages. Six hours after the start of the anaesthetic/oxygen exposure, animals were briefly exposed to isoflurane and immediately killed by rapid cervical dislocation and decapitated. The brains were quickly and atraumatically removed and the entire cortex was dissected and placed in Ringer's solution (155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM HEPES, 10 mM glucose) at 4 °C to wash away blood before being placed immediately into RNAlater (Life Technologies, Paisley, UK) to stabilise the RNA. All samples from individual rats were analysed separately and independently without pooling of samples.

2.2. RNA analysis

Total RNA was extracted from the cortex of each brain with TRIzol reagent (Invitrogen Ltd., Paisley, UK) according to the manufacturer's instructions. Total RNA was further purified using Qiagen column based methodology (Qiagen Ltd., Manchester, UK). The integrity of total RNA from each sample was established using an Agilent 2100 Bioanalyser and RNA 6000 Pico II Labchips® (Agilent Technologies UK, Cheshire, UK). Complementary RNA (cRNA) targets for hybridization were prepared and hybridized to the Rat 230 2.0 Genechip® for 16 h at 45 °C according to the Affymetrix protocol and identified using biotin-streptavidin-phycoerythrin labelling [10,11]. To control for hybridization, washing, and staining procedures, the array also contains probe sets for various non-eukaryotic cDNA transcripts which were added to the hybridization mix. Fluorescence patterns of the microarrays, which indicates gene expression, were scanned at 3 μm resolution using an Affymetrix gene array scanner and the digital images were analysed using Affymetrix Microarray Software Suite 5.0 and dChip™ version 2010 (http://www.dchip.org). Differential gene expression was determined by comparing data from isoflurane exposed rats to that seen in control rats exposed to oxygen only. Only genes which changed by at least 2 fold with a fluorescent intensity of >100 units were included ($P < 0.05$). In accordance with the recommended analysis strategy for small data sets, the results were permutated 1000 times and the false discovery rate was <15 out of ~30,000.

2.3. Data analysis methodology

2.3.1. Network analysis

Fluorescence data from each animal from both groups were mined using standard clustering techniques and principal component analysis. Essentially this means that genes having similar expression levels were grouped. Quantitative modelling of the direct interactions between genes was then performed using graphical Gaussian models that represent causal dependencies in biomolecular networks. We used the GeneNet software package which computes the direct interaction between pairs of genes by the partial correlation matrix [12]. The partial correlation of a given pair of genes is the correlation of the expression data for that gene pair conditioned on all the other genes; the partial correlation matrix is the matrix with all these pairwise partial correlations. The use of partial correlations allowed us to obtain, from multiple samples of gene expression data, a measure of the causal (direct) interactions between genes, as opposed to a simple correlation, which may have nothing to do with causation. The output of the GeneNet analysis is a graphical network where the nodes are genes, and the links represent genes that have strong interactions. This is a tried and tested method based on Bayesian probability principles, used successfully in previous gene expression studies to unravel metabolic networks in, for example, breast cancer data sets [13].

Further analysis was undertaken using techniques originally described for analysis of social networks [14,15]. There are various measures which are used to describe networks. Networks consist of *nodes*, which in this case are the genes, and each connection to another gene is called an *edge*; the number of edges connected to a single node is called the *degree* of the node. The topology of the network can be presented graphically where the direction of the interactions is identified (i.e. where a gene acts to increase or decrease expression of another). The thickness of the edges indicates the strength of the interaction. The *degree distribution* is how many nodes (genes) have a certain degree number. The 'importance' of each node can be described in several ways: the *degree centrality* is simply the degree of a node (i.e. the number of edges or interactions it has) and highly connected nodes with a high degree (5 or more) are called *hubs. Closeness centrality* is the average of the pathlength of one node to every other node such that the most important nodes have a low value, describing shortest paths to other nodes. *Betweenness centrality* is a measure of a node's influence as a 'gatekeeper' and is essentially the % of shortest paths that include a given node. Finally the *cohesion* of the network is the number of nodes which would need to be removed to render the network disconnected. A schematic diagram depicting the key features of an example network is shown in Fig. 1 and the various measures are described in Table 1.

2.3.2. Functional clustering

Differential gene expression between anaesthesia and control rats was clustered into functional groups using the Gene Ontology Enrichment Analysis Software Toolkit (GOEAST) [16]. For this, the gene names with known functions were grouped into key functional categories identified by accepted GO numbers to enable unambiguous groupings.

3. Results

3.1. Network analysis

The expression network diagrams shown in Fig. 2A and B shows the interactions between the genes. The networks consisted of 18 connected genes in the non-anaesthetized control rats (Fig. 2A) and 15 connected genes in the anaesthetised rats (Fig. 2B). The full names and functions of the proteins encoded by these genes are listed in Table 2. It can be seen that the network after anaesthesia (Fig. 2A) is completely different from that seen in the non-anaesthetized rats (Fig. 2B). Genes which are most highly connected have a high degree centrality and dominate the network topology –i.e. hub genes. Two hub genes (nodes), both with 6