Environmentally enriched pigs have transcriptional profiles consistent with neuroprotective effects and reduced microglial activity

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Abstract

Environmental enrichment (EE) is widely used to study the effects of external factors on brain development, function and health in rodent models, but very little is known of the effects of EE on the brain in a large animal model such as the pig. Twenty-four young pigs (aged 5 weeks at start of study, 1:1 male: female ratio) were housed in environmentally enriched (EE) pens and provided with additional enrichment stimulation (a bag filled with straw) once daily. Litter, weight and sex matched controls n= (24) were housed in barren (B) conditions. Behaviour was recorded on alternate days from study day 10. After 21 days, RNA-sequencing of the frontal cortex of male piglets culled one hour after the enrichment stimulation, but not those at 4 hours after stimulation, showed upregulation of genes involved in neuronal activity and synaptic plasticity in the EE compared to the B condition. This result is mirrored in the behavioural response to the stimulation which showed a peak in activity around the 1 hour time-point. By contrast, EE piglets displayed a signature consistent with a relative decrease in microglial activity compared to those in the B condition. These results confirm those from rodents, suggesting that EE may also confer neuronal health benefits in large mammal models, through a potential relative reduction in neuroinflammatory process and increase in neuroprotection driven by an enrichment-induced increase in behavioural activity.

1. Introduction

Environmental enrichment (EE) paradigms are widely used in laboratory rodents as a method to study the effects of environmental influences on brain development and function (1). EE utilises multiple methods of environmental modification to facilitate cognitive and motor stimulation in a species appropriate manner. In rodents this is often achieved through the use of novel objects, running wheels, nesting material and group housing (2).

The beneficial effects of EE on physiology and brain function in rodents are well documented (reviewed in (3)). EE paradigms have been shown to have a neuroprotective effect through the

38 inhibition of spontaneous apoptosis (4) and through increased cell proliferation and neurogenesis in
39 the hippocampus (5-7) and prefrontal cortex (8). Indeed, EE has been shown to limit
40 neurodegeneration in models of aging (9, 10), and can rescue some behavioural and physiological
41 brain effects of early life stress (11, 12). Current evidence suggests this is most likely precipitated by
42 the mechanisms underlying neuronal plasticity, due to increased expression of trophic factors and
43 immediate early genes (IEG) in the brains of EE animals (13-15).
44 Expression of neurotrophins has also been proposed as a contributory factor in mood disorders, in
45 particular stress-induced depression and anxiety. While the main focus has been around expression
46 of brain derived neurotrophic factor (BDNF) (16-18), there are a number of other trophic factors and
47 IEGs with a role in stress resilience and mood. Neuronal growth factor (*Ngf*) mRNA has been shown
48 to be reduced in the dentate gyrus in response to restraint stress in rats (19). Environmental
49 deprivation during early life in rats reduces IEG expression in the prefrontal cortex both as a chronic
50 effect (20), in response to social interaction (21), and after chronic social defeat stress in mice(22).
51 Levels of IEG *Arc* mRNA in the frontal cortex and hippocampus of rats exposed to predator scent
52 stress were reduced in those animals which displayed an extreme anxiety-like behavioural response,
53 and in the dentate gyrus *Arc* mRNA expression levels also correlated with levels of circulating
54 corticosterone(23).
55 Interestingly, depressive-like behaviour in rodents induced by social isolation in early life not only
56 results in decreased expression of a number of IEG involved in synaptic plasticity, including *Bdnf, Egr-*
57 *1* and *Arc* ((24), mice), but also in increased microglial activation ((25), rats). Microglia are specialised
58 macrophages of the central nervous system that play a role in neuromodulation, phagocytosis and
59 inflammation (reviewed in(26)). There is increasing evidence to suggest that microglial mediated
60 brain inflammation is a contributing factor to a number of mental health and nervous system
61 disorders, such as multiple sclerosis(27), Alzheimer's disease(28), schizophrenia (29, 30), and major
62 depression(31). While the exact mechanism by which microglia contribute to these disorders is not
63 yet known, psychological stress has been shown to result in increased levels of microglia in the

64 prefrontal cortex of rats (32) while early life stress, in the form of maternal deprivation, has also been
65 shown to lead to increases in microglial motility (33) and phagocytic activity (34) that persist into
66 adulthood. Anti-inflammatory drugs such as minocycline (which blocks microglial activation) produce
67 antidepressant effects in rodent models (35). Several anti-psychotic medications have also been
68 shown to block microglial activation (36, 37).
69 The physiological and behavioural effects of a number of nervous system disorders can be modulated
70 by EE in rodent models (38), and EE is now widely used as an experimental tool in studies of
71 neurodegeneration and neural development (39). Due to its beneficial effects on behaviour, EE has
72 long been used as a method to improve the welfare of production animals, by providing
73 environmental manipulations that allow the animal to behave in a more species-typical way
74 (reviewed in (40)). However there has been little work on the effects of EE on the brain in a large
75 animal model, such as the pig. The aim of the present study was to investigate the effects of EE on
76 gene expression within the frontal cortex of young pigs with the aim of identifying potential gene
77 expression signatures of a healthy brain in a large animal model, and to potentially identify early
78 signatures of neural ill-health in the absence of abnormal behaviours.
79

80 ## 2. Materials and Methods

81 ### 2.1 Ethical review

82 All work was carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986 under
83 EU Directive 2010/63/EU following ethical approval by SRUC (Scotland's Rural College) Animal
84 Experiments Committee. All routine animal management procedures were adhered to by trained
85 staff and health issues treated as required. All piglets remaining at the end of the study were
86 returned to commercial stock.

87 ### 2.2 Animals and general experimental procedures

88 Post-weaning behavioural observations were carried out on litters from six commercial cross-bred
89 mothers (Large White x Landrace); the boar-line was American Hampshire. Artificial insemination
90 was performed using commercially available pooled semen. Litters were born within a 72 hour time
91 window in free-farrowing pens which allowed visual and physical contact (nasal contact through
92 bars) with neighbouring litters. No tooth resection was performed and males were not castrated. In
93 line with EU Council Directive 2008/120/EC tail docking is not routinely performed. Weaning
94 occurred at 24-27 days of age. At weaning piglets were weighed, vaccinated against Porcine
95 Circoviral Disease and ear tagged for identification. Post-wean diet was in the form of pelleted feed
96 (Primary Diets Prime Link Extra) provided *ad libitum*. At 4 days post weaning, 8 piglets per litter were
97 selected as being as close to the litter average weight as possible while balancing for sex (1:1 male to
98 female). These 8 piglets per litter were split into sex balanced quartets to give final group sizes of 4
99 piglets per matched group. One group was then housed in an enriched (EE) pen and one in a barren
100 (B) pen, all in the same room (Figure 1). Post weaning pens did not allow for physical or visual contact
101 between neighbours. All pens contained feed troughs ~80cm in length and piglets had access to one
102 water nipple per pen.
103
104 All piglets were marked on the back with spray marker paint to allow individual identification. B pens
105 measured 1.8m x 1.8m with concrete floors and rubber matting (1.8m x 1.2m) in one corner to
106 provide some thermal insulation. Kennels were provided for the first 10 days to enhance thermal
107 comfort as B housed piglets had no opportunity to use straw to control their thermal environment as
108 the EE pigs could. EE pens measured 3.6m x 1.8m also with concrete flooring and with large
109 quantities of straw covering the floor (covering half the pen to a depth of 0.15m). The same rubber
110 matting was also provided in the enriched pens. Piglets in the six EE pens were provided with
111 additional enrichment once daily (enrichment stimulus) in the form of a plastic bag (approximately
112 400mm x 750mm x 150mm) filled with straw (approximately 2kgs). This bag was placed in the EE
113 pens after morning husbandry was complete (at around 10 am). At the same time the gate of the

matched B pen was approached to account for the stimulating effect of human presence. The bag was removed at the end of each day (~1700 hours) and a new bag used on the following day. The bag was open at one end to allow straw to be extracted by the piglets and was the only source of fresh straw given to the EE pigs daily. The bag was first introduced at day 5 post weaning and thereafter daily. Pen cleaning and enrichment order was randomised daily. In accordance with the Defra Code of Recommendations for the Welfare of Livestock(41), temperature within the room was automatically controlled at 22°C and artificial lighting was maintained between the hours of 0800 to 1600, with low level night lighting at other times.

2.3 Behavioural observations

Piglets were digitally recorded in their home pen using Sony LL20 low light cameras with infra-red and a Geovision GV-DVR. Two cameras were set up per EE pen, one at the rear and one at the front to provide maximal coverage, and one camera per B pen. Piglets were observed in their home pens on five days between the ages of 41 and 53 days (+/-3 days due to staggered farrowing times). Piglets were all observed on the same days during the same time periods to negate any time of day effects on sampling. The behaviour scores are the cumulative total of how many times that behaviour occurred in the timeframe within the pen. Observations began after morning husbandry was complete, 15 minutes prior to the time point when the additional enrichment was provided to the EE pigs, and finished 4 hours after the provision of additional enrichment. Behaviours were recorded by scan sampling with a frequency of one minute(42) for the total 4.25hour duration. The observer used a 10 second window to aid in accurate identification of the behaviour occurring at the start of every scan sample. A condensed ethogram based on previous work in enrichment (43) and play (44) in pigs (Table 1) was used. All behaviours were recorded as frequencies. One observer completed all video analysis to remove any reliability issues relating to multiple observers.

138 **Table 1:** The ethogram used for behavioural scoring. Individual postures were considered inactive
139 behaviours and all other behaviours as active as when active the posture is included in the active
140 behaviour description and not scored separately.

Category/Behaviour	***Definition/type***
	Active Behaviours
Social	
Belly nosing penmate	A distinctive, rhythmic up-and-down movement of a piglet's snout against the belly of another piglet. Piglets may be standing, sitting or lying when preforming or receiving belly nosing
Tail biting	Manipulation, sucking or chewing tail of pen mate
Locomotion (without object or social)	
Walking	A steady 4 beat gait with 2 or 3 legs bearing weight at any one time (depending on the phase of the movement).
Running	Energetic running and hopping in forward motions often associated with excitability, using large areas of the pen, occasionally coming into marginal/accidental contact with other piglets
Hop/flop/pivot	High energy movements on the spot in an upwards (hop), downwards (flop) or circular (pivot) direction but with little or no forward propulsion
Object and pen Interaction	
Object interaction_enrichment stimulus	Any active interaction with the enrichment stimulus. Piglet may be locomotory or stationary. Includes biting/chewing of bag, walking or running with bag, climbing in bag, shaking bag vigorously and lying on bag.

Object interaction_no enrichment stimulus	Active interaction with other pen components, such as straw, rubber matting and shavings. As above may include walking or running with the object, and chewing/biting object
Nosing/exploring pen	Piglet walking/running or stationary with nose to floor or pen walls performing a gentle nosing motion
Feeding/drinking	Piglet oriented with snout in trough/drinker appearing to be chewing or swallowing
Inactive Behaviours	
Postures	
Lateral lying	Piglet lying on its side
Ventral lying	Piglet lying on its front
Sitting	Piglet sitting on its haunches with front legs straight
Standing	Piglet standing still. Not performing any other visible behaviour
Other	
Not visible	Piglet out of view of camera

2.4 Statistical analysis of behaviour

The full dataset was condensed to give one data point for each behaviour per hour period per day for each pen being the cumulative recordings for all piglets in that pen in that observation period. The introduction of the bag is time-point 0. Analysis was performed on these mean values for each period per pen averaged across the 5 observation days. Analysis of the behavioural data was performed in Minitab version 17.3 (2016) using a generalized linear model with treatment as a fixed effect and time point as a random effect. Sex, litter of origin and pen were included in the model as covariates for the behaviour (and weight data).

2.5 Tissue collection

151 At 54 days of age (+/- 2 days) one male piglet per pen was sedated at one hour after the introduction
152 of the additional enrichment stimulus, and one male per pen at 4 hours post enrichment stimulus.
153 An intramuscular injection was used with a combination of Domitor (1mg/ml medetomidine
154 hydrochloride) (0.01ml/kg), Ketamine (0.1ml/kg), Hypnovel (5mg/ml medazolam) (0.1ml/kg) and
155 Stresnil (40mg/ml azaperone) (0.025ml/kg) (as previously described in 43). Piglets were left for 15
156 min to allow sedation to take full effect before being euthanized via intercardial injection with
157 Euthatal (200mg/ml sodium pentobarbital) (0.7ml/kg) for brain tissue collection. Male brain tissue
158 was sampled to be consistent with other EE studies, mainly performed in rodents. Piglet brains were
159 removed whole and dissected over dry ice. For continuity all dissections were performed by a single
160 researcher using http://www.anatomie-amsterdam.nl/sub_sites/pig_brain_atlas for reference,
161 utilising both parasagittal and rostrocaudal views. Frontal cortex was placed in RNAlater (Ambion)
162 and agitated on a tube rotator for 15 minutes to assist in the perfusion of RNAlater through the
163 tissue. Samples were frozen at -20˚C until required.

164 **2.6 Preparation of tissue samples**

165 Frontal cortex samples in RNAlater were removed from -20˚C and allowed to reach room
166 temperature. RNA extraction was performed using the Qiagen RNeasy lipid tissue kit as per
167 manufacturer's instructions. 80mg of tissue was used from each sample and a TissueRupter (Qiagen)
168 used for homogenization. Extracted RNA was stored at -20˚C until required (<3months). Samples
169 were quantified and quality checked using a 2200 Tapestation (Agilent).

170 **2.7 RNA-seq and differential expression analysis**

171 Poly A+ library preparation and RNA sequencing (RNA-seq) of the prepared samples was carried out
172 by Edinburgh Genomics Next Generation Sequencing Service
173 (http://genomics.ed.ac.uk/services/sequencing) using the Illumina HiSeq 4000 platform, generating
174 >30 million strand-specific 75bp paired-end reads per sample. RNA-seq reads were processed using
175 the high-speed transcription quantification tool Kallisto(45), version 0.42.4, generating transcript-
176 level expression estimates both as transcripts per million (TPM) and estimated read count. Kallisto

177 quantifies expression by building an index of k-mers from a set of reference transcripts and mapping
178 the reads to these directly. For this purpose, we used the combined set of cDNAs and ncRNAs from
179 annotation Sscrofa11.1 (ftp://ftp.ensembl.org/pub/release-
180 90/fasta/sus_scrofa/cdna/Sus_scrofa.Sscrofa11.1.cdna.all.fa.gz and
181 ftp://ftp.ensembl.org/pub/release-90/fasta/sus_scrofa/ncrna/Sus_scrofa.Sscrofa11.1.ncrna.fa.gz,
182 respectively, accessed 1st November 2017). Transcript-level read counts were then summarised to
183 the gene level using the R/Bioconductor package tximport v1.0.3 (46), with associated HGNC gene
184 names obtained from Ensembl BioMart v90 (47). The tximport package collates Kallisto's output into
185 a count matrix, and calculates an offset to correct for differential transcript lengths between samples
186 (the consequence of differential isoform usage). The count matrix was imported into the
187 R/Bioconductor package edgeR v3.14.0 (48) for differential expression analysis, with the 'trimmed
188 mean of M values' method used to normalise gene counts. Genes with expression level estimates
189 lower than 2 TPM in one or both treatment groups were filtered out as noise. After filtering lowly
190 expressed genes, the final dataset comprised 21,971 genes. Genes were considered to be
191 differentially expressed (EE vs B) when they returned a between group fold-change greater than +/-
192 1.2, with a *p*-value <0.02. These thresholds for fold change and p-value were chosen specifically for
193 this study, to minimise noise with regard to identifying a conservative set of significantly
194 differentially expressed genes. A fold change of +/- 1.2 reduces the likelihood of natural variation in
195 expression (i.e. which would be observed around a fold change of 1), as does an alpha level more
196 stringent than the conventional 0.05. The sequencing data generated for this project is deposited in
197 the European Nucleotide Archive (ENA) under study accession number PRJEB24165
198 (http://www.ebi.ac.uk/ena/data/view/PRJEB24165).

199 **2.8 Gene set and gene signature enrichment analysis**

200 Gene set enrichment analysis was performed on the differentially expressed gene lists using
201 Panther.db v13.0 (release 20170413) with default parameters. To assess the statistical significance of
202 enrichment-induced variation in gene expression for small groups of genes with a common function

203 ('signature genes'), we used a randomisation test as in (49). Two sets of gene signatures, derived
204 from previous human studies, were first obtained for IEG genes (e.g. (50-58)) and microglial genes
205 (59) (Table S4), and filtered to remove those genes not annotated in pigs (resulting in n = 123 and
206 569 genes, respectively). Subsets of x genes were drawn at random s = 10,000 times from the set of
207 all genes for which a fold change is quantified (i.e. for each comparison of treatment groups), where
208 x = number of signature genes. We calculated q, the number of times the set of signature genes had
209 a higher (for IEG genes) or lower (for microglial genes) median fold change than each randomly
210 chosen subset. Letting $r = s-q$, then the p-value of this test is $r+1/s+1$.

211 **2.9 Network analysis of transcriptomic changes**

212 These expression values were entered into the network analysis tool Graphia Professional (formerly
213 Miru, derived from BioLayout *Express*^{3D} (60, 61); http://kajeka.com/graphia-professional). A sample-
214 to-sample analysis was performed using a correlation coefficient threshold of 0.99 to assess the
215 impact of individual variation on all gene expression profiles. A gene-to-gene network was also
216 created from the whole dataset using a correlation coefficient threshold of 0.90. The nodes (genes)
217 were clustered using the Markov Clustering (MCL) algorithm (62) with an inflation value (which
218 determines cluster granularity) of 2.2. This algorithm identifies tightly coordinated sub-structures
219 within the overall network.

220

221 **3. Results**

222 **3.1 Additional enrichment stimulation increases active behaviour**

223 An analysis of the percentage of active behaviours in EE vs. B housed piglets found a treatment by
224 time-point effect ($F_{4,43}$=4.2, p=0.005). The coefficients show this difference to be attributable to an
225 increased proportion of active behaviours in the EE piglets at one hour post provision of the
226 enrichment stimulus (t_4=3.99, p<0.001)(Figure 2). Piglet sex was not observed to have an effect on
227 the active behavioural response to enrichment.

228

229 A housing treatment x time effect was observed in locomotor (without object or social) behaviour
230 ($F_{4,43}$=3.19, p=0.021), interaction with the enrichment stimulus ($F_{4,43}$=59.42, p<0.001) and interaction
231 with other pen components, such as rubber matting ($F_{4,43}$=2.75, p=0.038). These effects peaked at
232 one hour post provision of the enrichment stimulus to the EE pens with greater performance of
233 locomotor (without object or social) behaviour (t_4=2.75, p=0.008) and interaction with the
234 enrichment stimulus (t_4=15.08, p<0.001), and at two hours post enrichment stimulus for interaction
235 with other pen objects (t_4=2.07, p=0.04) in the B pens. A greater proportion of lateral lying in B
236 housed pigs was observed at one hour post enrichment stimulus (t_4=2.69, p=0.01). An effect of
237 housing treatment alone was observed in nosing of pen-mates ($F_{1,47}$=25.9, p=0.007) with more nosing
238 of pen-mates occurring in the B housed piglets. An effect of time alone was found in feeding
239 behaviour ($F_{4,43}$=37.13, p=0.002), nosing of the pen ($F_{4,43}$=11.47, p=0.018) and a trend towards lateral
240 lying ($F_{4,43}$=4.3, p=0.09). No effect of time or treatment was observed in any of the other behaviours
241 recorded. Means and S.E.M for behaviours are shown in table S1.

242

243 There was no difference between piglet growth (as measured by Average Daily Gain in weight, ADG)
244 during the pre-weaning period, prior to the start of the study ($F_{(1, 47)}$ = 0.027, p = 0.873). Piglet
245 growth was observed to differ between environments ($F_{(1, 47)}$ = 5.68, p = 0.038) during the study
246 period, with EE piglets having on average a higher ADG than B piglets (EE mean = 688.9g SEM = 15.5 ,
247 B mean = 654.0g SEM = 12.6).

248

249 **3.2 Transient up- and downregulation of genes in response to environmental enrichment**

250 Complete lists of differentially regulated genes are provided in Table S2. Initial analysis revealed that
251 for a large majority of genes, expression in the frontal cortex was unaffected by the environmental
252 enrichment (Figure 3).

253

When all 21,971 genes were included in a network analysis (see Materials and Methods), members of the same litter showed similar expression patterns, regardless of treatment or time. This was particularly noticeable for litters L3, L4 and L6 (Figure 4A). Using the full gene set there was no association with treatment (Figure 4B) or time (not shown).

A set of 349 genes were up-regulated after one hour of environmental enrichment but appeared to return to baseline after four hours (Table 2). A second set of genes was down-regulated at both one hour and four hours (Table 2) in the EE cohort. The composition of these two gene sets is discussed below.

Table 2: Numbers of genes differentially expressed in the frontal cortex of piglets housed in EE and B environments at one and four hours post enrichment. The last two rows combine all piglets within each treatment regardless of time-point.

Comparison	Direction of Change	# Differentially Expressed Genes
1hr EE vs. 1Hr B	Up	357
	Down	652
4hr EE vs. 4hr B	Up	46
	Down	83
All EE vs. All B	Up	125
	Down	184

Gene set enrichment analysis at one hour post enrichment highlights an over-representation of genes involved in synaptic transmission being up-regulated in the EE animals (Table 3), with an over-

272 representation of genes involved in apoptosis and cellular defence being down-regulated in the EE
273 animals. Further analysis of the gene lists revealed that approximately 8% of those genes
274 upregulated at 1 hour in the EE animals are known IEGs while 15% of those genes down-regulated in
275 the EE (up-regulated in B) animals at 1 hour are related to microglial processes. At four hours post
276 enrichment no gene sets were found to be overrepresented using the Panther GSEA
277 overrepresentation test.
278
279 Significant *p*-values from randomisation tests (up-regulated IEG signature *p*=9.99E-05, down-
280 regulated microglial signature *p*=9.99E-05) would suggest these signatures do not occur by chance in
281 EE animals.
282
283 **Table 3:** Panther GO-Slim Biological Process GO terms statistically overrepresented in the one hour
284 EE vs one hour B comparisons. The 'direction of change' column signifies the direction of differential
285 gene expression in the EE animals relative to the B animals. The observed column is the number of
286 genes assigned this GO term in the submitted gene list; the expected column is the number of genes
287 expected given the full *Sus scrofa* reference list.

Comparison	Direction of Change	GSEA Overrepresentation Test (PANTHER release 20170413)				
		GO Terms	Observed	Expected	Fold Enrichment	*p*-value
	Up	Synaptic transmission	15	4.41	3.4	1.12E-02
		System development	14	4.45	3.14	4.53E-02
		Regulation of molecular function	16	5.30	3.02	2.54E-02

1hr EE vs. 1Hr B		Regulation of phosphate metabolic process	18	6.34	2.84	2.05E-02
	Down	JAK-STAT cascade	8	0.61	13.16	6.07E-05
		Negative regulation apoptotic process	16	2.83	5.65	1.15E-05
		Cellular defense response	14	2.64	5.3	1.68E-04
		Cell Adhesion	24	7.98	3.01	6.52E-04
		Immune system process	21	7.02	2.99	2.93E-03

In a network analysis using only the differentially expressed genes, the EE samples were close together in a relatively tight group, while the B samples were more dispersed, but still located together with little overlap with the EE samples (Figure 4). In addition there was no relationship with litter, indicating that for the differentially expressed gene set the effect of environment was stronger than any intrinsic difference based on genetics or early life (e.g. maternal) effects (Figure 5).

The expression profiles of the differentially expressed genes were analysed to create a gene-to-gene network. As might be expected (as these were differentially expressed in both directions) there were two clear elements in the network, comprising one element where the overall gene expression level was higher in the EE animals and a second element where the level was lower in EE animals. Similar results were seen when using the one hour, four hour or complete set of differentially expressed

genes (Figure S1: Panel A-C segregated gene cluster histograms by animal, Panel D Clusters for all animals at 1 hour).

When the DEGs at one hour were used to construct a network graph, cluster 1 (which contains the majority of genes with average expression reduced in the EE condition) contained microglia-associated genes such as *CSF1R* (63), *IRF8* (64) and *P2RY12* (65) alongside various connective tissue genes including several collagen genes and *FBN1.* Cluster 4, where the average expression was increased in EE samples, contained IEGs such as *ARC,* several EGR genes, *FOS* and *IER2*. Cluster 2 contained largely neuronal genes which were not differently expressed between environments. Cluster 3 contained additional connective tissue genes with high levels in four of the B individuals. Clusters 5 and 6 represent groups of genes that were increased in a single individual. The gene lists for each cluster and average expression profiles for clusters where treatment differences were shown are presented in Table S3 and Figure S1.

The profiles for the four hour samples were largely determined by high or low expression in a single or small number of individuals. Many of the genes in this list were non-coding RNAs or unannotated genes. When both time points were included in the analysis, cluster 3 primarily contained IEGs and showed a pattern in which the one hour EE samples had higher expression than the one hour B samples, but at four hours the two groups had similar expression. One individual in the B group had a spike of IEG expression at four hours although most of the four hour B samples showed lower IEG expression than the EE samples.

4. Discussion

The EE piglets in this study displayed more active behaviour (combining locomotion (without object or social), social and object interaction) than B piglets, but only in the first hour after the additional

327 enrichment stimulus (bag filled with straw) was provided. This was not affected by sex. This suggests
328 that the piglets' behavioural response to enrichment in this experiment was largely defined by the
329 period that the additional enrichment stimulus remained novel. After the 4 hours of observation the
330 piglets had tended to remove some or all of the straw from the enrichment stimulus and reduced the
331 physical integrity of the bag which may have reduced the novelty value of the stimulus. Despite the
332 larger space allowance and fresh bedding (straw, given as part of the additional enrichment stimulus)
333 provided daily to the EE piglets, their overall activity profile was not different to the B housed piglets
334 outside of the first hour post additional enrichment (using a scan sampling methodology). Overall
335 total activity levels were lower in this study than in previous studies (see (66) for a review), but the
336 proportion of active time spent interacting with the enrichment stimulus or the pen environment
337 was higher than previously reported. Within the time spent active there were some effects of
338 housing on specific behaviours. B housed piglets displayed more nosing of pen-mates (primarily
339 nosing of the belly of other pigs) over the course of the observation, not dependent on time. This
340 maladaptive behaviour is known to occur more frequently in piglets when the opportunities to use
341 the snout to forage and explore are not provided(67). Despite no differences in time spent feeding
342 being observed between treatments, piglets in the EE environment experienced higher growth rates
343 than those in the B environment. Enriched housing has previously been shown to increase body
344 weight and growth rate in grower pigs but not in piglets at such a young age (68). A recent meta-
345 analysis has found that the provision of straw bedding is a significant factor in the increased average
346 daily gain observed in EE housed pigs (69) though there has been little research to determine why
347 this may be the case. In rodents, increased brain weight in the absence of increased body growth has
348 been observed in environmentally enriched conditions (reviewed in (70)). Given the short time frame
349 of this study we would not expect to observe significant differences in brain weight however over a
350 longer period this may well be a contributory factor to observed weight gain.
351

352 The gene expression changes observed in this study mirror those of the behavioural differences, with
353 most genes differentially expressed at one hour post the provision of the additional enrichment
354 stimulation and few genes differentially expressed at four hours post his point. The observed
355 increase in genes involved in synaptic transmission at the one hour time point in EE animals supports
356 previous work describing a transient increase in cell activity in the hippocampus of rats following
357 environmental enrichment (reviewed in (71)). This increase in synaptic transmission is likely to be the
358 mechanism by which EE leads to increased brain weight (72), greater cortical depth (73), increased
359 neurogenesis (6, 74) and increased synapse formation (75). The set of transiently-induced genes at
360 one hour shows significant overlap with recent curated sets of IEGs defined in stimulated neurons
361 based upon single cell RNA-seq (76, 77). Increases in expression of neurotrophic factor proteins
362 (neuronal growth factors) have previously been observed in multiple brain regions of rats housed in
363 enriched cages (14) and were observed as gene expression changes in the frontal cortex of the
364 piglets in the current study, although not all growth factors fall within the gene set enrichment
365 analysis. Neuronal growth factors (such as *NGF, BDNF* and *VGF,* all of which were upregulated in this
366 sample set) are considered to be regulators of cell growth and survival in both the developing and
367 adult nervous system (reviewed in(78)) and have been shown to acutely potentiate synaptic plasticity
368 (79, 80).
369 Experience dependant plasticity, the way by which synaptic connections are strengthened or
370 weakened, or indeed formed or eliminated, in response to experience is a well established
371 mechanism (reviewed in(81)). Intracellular scaffold protein and regulator of membrane trafficking
372 GRASP (mRNA upregulated in the EE pig frontal cortex) has multiple protein interacting domains
373 allowing a wide range of protein assemblies to be trafficked at the synapse (82). GRASP is known to
374 be involved in dendritic outgrowth (83) and in synaptic plasticity through its interaction with group I
375 and II metabotrophic glutamate receptors (84). Reductions in GRASP protein expression have been
376 observed in post mortem prefrontal cortex of schizophrenia patients (85) with no correlation to
377 illness duration or history of medication. Similarly, decreases in mGluR2 have been observed in

378 multiple psychiatric conditions including schizophrenia (86) and addiction (87), while treatment with
379 the antidepressant medication imipramine has been shown to increase mGluR2 protein expression in
380 the hippocampus of wild-type rats (88) but not to increase mRNA expression. Another G-protein
381 coupled receptor upregulated in the EE pigs, GPR3, has been shown to be involved in anxiety related
382 behaviours, with *Gpr3-/-* mice displaying increased anxiety-like behaviours that could be rescued
383 with the use of the anxiolytic diazepam (89). Upregulation of these G-protein coupled receptors and
384 their targets may suggest that EE piglets in the current study were experiencing reduced levels of
385 anxiety and a more positive affective state compared to their B counterparts. Given the lack of sex
386 difference in behavioural activity observed in this study, and no evidence of overlap between our list
387 of DE genes and those shown to be sexually dimorphic in neonatal rodent cortex (90), we would
388 propose that this effect would be consistent across both males and females.
389

390 An unexpectedly large proportion of the set of genes downregulated in the brains of the pigs
391 exposed to environmental enrichment (relative to those in the B environment) are clearly associated
392 with microglia. Numerous recent studies have identified microglia-associated genes in mice and
393 humans (e.g. (59, 91-94)) however there has been little work focused on the pig. The set of genes
394 down-regulated after one hour post the additional enrichment stimulation included generic myeloid
395 markers such as *PTPRC* (*CD45*) and *CD68*, as well as most well-documented microglial markers (*C1QA,*
396 *MYD88, P2RY12, TGFBR2, TREM2*). These findings suggest that microglia have altered transcriptional
397 activity in EE relative to B pigs. With our study design it is impossible to determine whether the EE or
398 B animals are the baseline so any changes in expression are relative, however irrespective of whether
399 microglial activity is greater in B or reduced in EE animals, this result suggests that a relative
400 reduction in activity may have benefits for brain health (95, 96). While reduced transcriptional
401 activity could arise due to a selective reduction in microglia number, in rodents the number of
402 microglia is relatively stable over time (97). Experimental depletion of microglia in rat neonates
403 results in lifelong decreases in anxiety-like behaviours and increased locomotory behaviour (98),

404 while increases in microglial numbers and activation states have been observed in stress-induced
405 depression models (reviewed in (31)). In human patients, increased microglial activation has been
406 observed using PET scanning in major depressive disorder (99), and from histology samples from
407 brains of depressed suicides (100). This suggests that B housed animals may be experiencing greater
408 anxiety and/or lower mood than their EE housed counterparts. This may provide part of the
409 functional link as to why pigs housed in enriched environments display more optimistic judgement
410 bias than those in barren conditions (101). Current evidence suggests that microglial gene expression
411 is not sexually dimorphic at this equivalent developmental stage (102), and thus while only male
412 brains were sampled in this study this may be extrapolated to include females with relative
413 confidence.
414 Interestingly, at the four hour time-point there is a high proportion of 5s ribosomal and spliceosomal
415 RNAs identified as differentially expressed (29% of the down regulated gene list, compared with 0.3%
416 at one hour), which may be adding noise to the dataset in the later time-point. As observed in the
417 network analysis, inter-individual variability in gene expression was greater at 4 hours, perhaps due
418 to the varying rates of decay of the effect of the additional enrichment stimulus, or due to the
419 animals no longer having a behavioural 'focus' (which may also explain the greater variability in gene
420 expression between B individuals than between EE individuals). This is consistent with the behaviour
421 analysis, which indicated that the benefit from the additional enrichment experience (bag of straw)
422 was only felt while the object remained novel. This may contribute to the noise in the data at four
423 hours, as animals may 'cope' with the lack of behavioural stimulation in different ways, or may be
424 being stimulated by other factors within the home pen (such as other interactions with pen mates).
425
426 The current study would suggest the benefits of enrichment may occur in a pulse-like fashion, with
427 bursts of behavioural activity being followed by a return to behavioural baseline, these bursts
428 mirroring the active engagement with the behavioural opportunities offered by the additional
429 enrichment stimulus. It would be of great interest to determine if these bursts of activity (and of

430 related differential gene expression) alter the structure and function of the brain in a way that
431 facilitates long term developmental change. Confirmation of the longer term effects of EE on
432 microglial numbers and activation are required to determine if the observed gene expression
433 changes are an indicator of lower neuronal health in B housed animals or if they are transient
434 expression changes with little long term consequence.
435

436 ## 5. Conclusions

437 In this study, behaviour was mainly organised around the time point when a daily enrichment
438 stimulus was provided. This resulted in a 'pulse' of active behaviour that was mirrored by temporal
439 changes in gene expression profiles in the frontal cortex. These gene expression changes primarily
440 affected genes involved in synaptic plasticity, neuroprotection and the immune response, with a
441 substantial fraction (15%) of known microglial signature genes showing relative down-regulation with
442 enrichment. Our analyses suggest that relative to piglets in barren environments, those in enriched
443 environments may experience reduced anxiety, increased neuroprotection and synaptic plasticity,
444 and an immune response consistent with reduced inflammatory challenge.
445
446

447 ## 6. Acknowledgements

448 We would like to acknowledge funding support for this work from the BBSRC Strategic funding to The
449 Roslin Institute, and from the Scottish Government's Rural and Environment Science and Analytical
450 Services Division (RESAS). Technical support was provided by the SRUC technicians Marianne Farish
451 Agnieszka Futro, Jo Donbavand and Mhairi Jack. Farm manager Peter Finnie provided general animal
452 husbandry assistance throughout the experimental period. Behavioural data collection was
453 performed by summer student Manon Poux and initial expression analysis by Edinburgh Genomics
454 bioinformatician (Dr Frances Turner).

455

456 7. **References**

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706

8. Figure Legends

Figure 1: Diagram of the layout of the experimental room. Double lines signify the outer room doors used for access. Grey circles indicate the location of cameras and filled black boxes show the location of feeders. Litter of origin is identified as L1-L6. Empty pens are indicated by hatched lines. EE pens were twice the size of B pens as indicated in the diagram. All pens were solid floored. EE pens had the addition of straw over two thirds of the flooring.

Figure 2: Percentage of behaviours classified as 'active' at each time point for EE and B piglets. Values are means with error bars indicating SEM. 60 scans per pen contribute to each time point after the addition of the bag (at time 0) and 15scans per pen in the pre period. Active behaviours include locomotion (without object or social) (running, walking, hopping, pivoting and flopping), object interaction (with enrichment, i.e. bedding or enrichment stimulus, or components of pen) and nosing (of pen-mates or pen structure). X-axis shows the time relative to the provision of the additional enrichment stimulus.

Figure 3: Differentially expressed genes. (Panel A) Largely similar expression profiles of frontal cortex in barren (x-axis) and enriched (y-axis) environments, with the exception of a subset of differentially expressed genes. Each point is a gene, with expression level measured as the median transcripts per million (TPM) across all replicates. The line y = x is shown in red. Differentially expressed genes are more distant from this red line. (Panel B) Volcano plot of the fold change in expression for all genes. X axis shows log2 fold change; Y axis shows –log10 *p*-value. Few genes demonstrate large fold changes, with most clustering at the root of the graph.

Figure 4: 3D network graph of all genes expressed in the frontal cortex. Each node (sphere) in the network graph represents a sample (individual), and each edge (line) a Pearson's correlation between samples ≥ 0.99. The local structure of the graph shows that gene expression profiles cluster

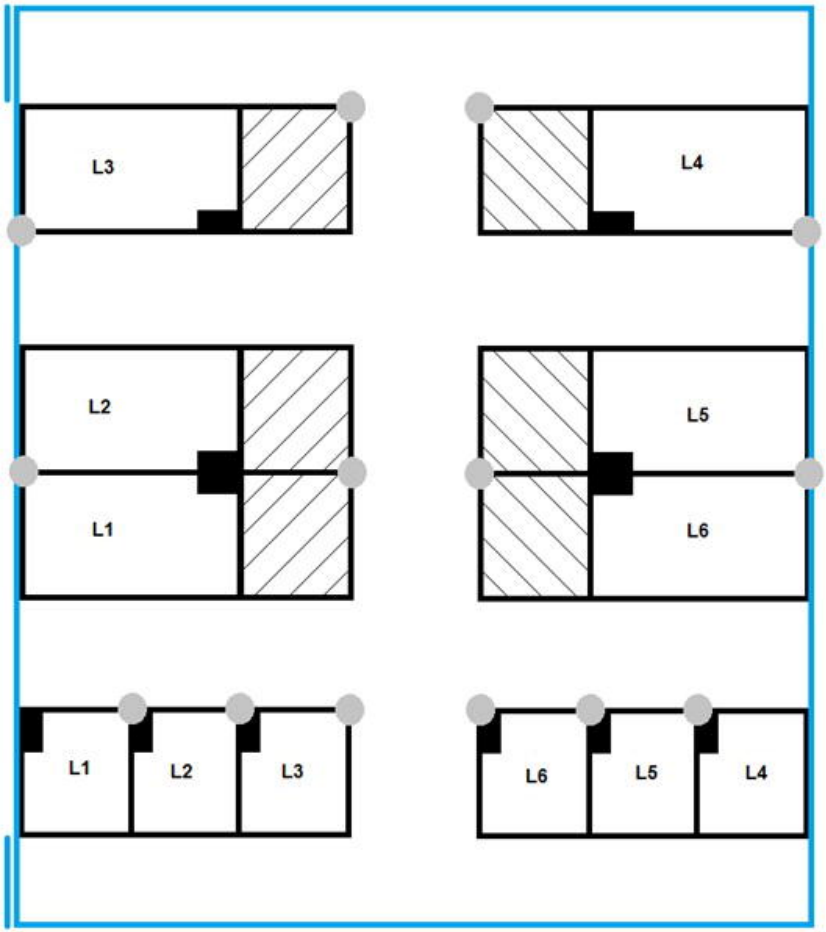
733 according to litter of origin (Panel A, where nodes are coloured by litter) and that there is little effect
734 of treatment (i.e. no clear clustering) upon overall gene expression (Panel B, where nodes are
735 coloured by environment).
736
737 **Figure 5:** 3D network graph of differentially expressed genes in the frontal cortex. Each node (sphere)
738 in the network graph represents a sample, and each edge (line) a Pearson's correlation between
739 samples ≥ 0.93. There is no clustering of gene expression profiles based on litter of origin (Panel A)
740 and an unambiguous effect of treatment on gene expression (Panel B). Inter-individual variability in
741 expression profiles appears lower in the EE pigs than in the B pigs.
742
743

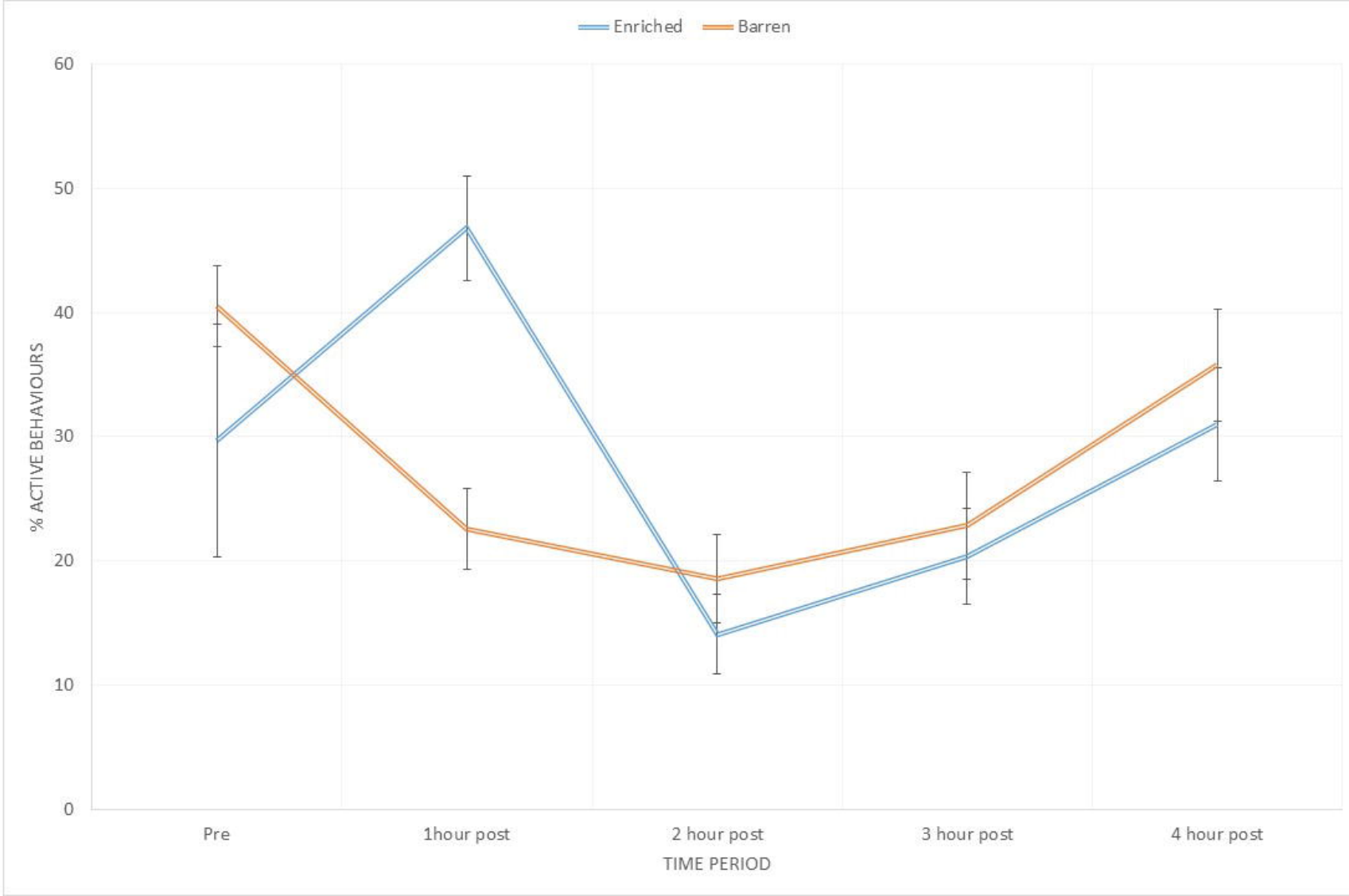
744 **9. Supplemental Information**

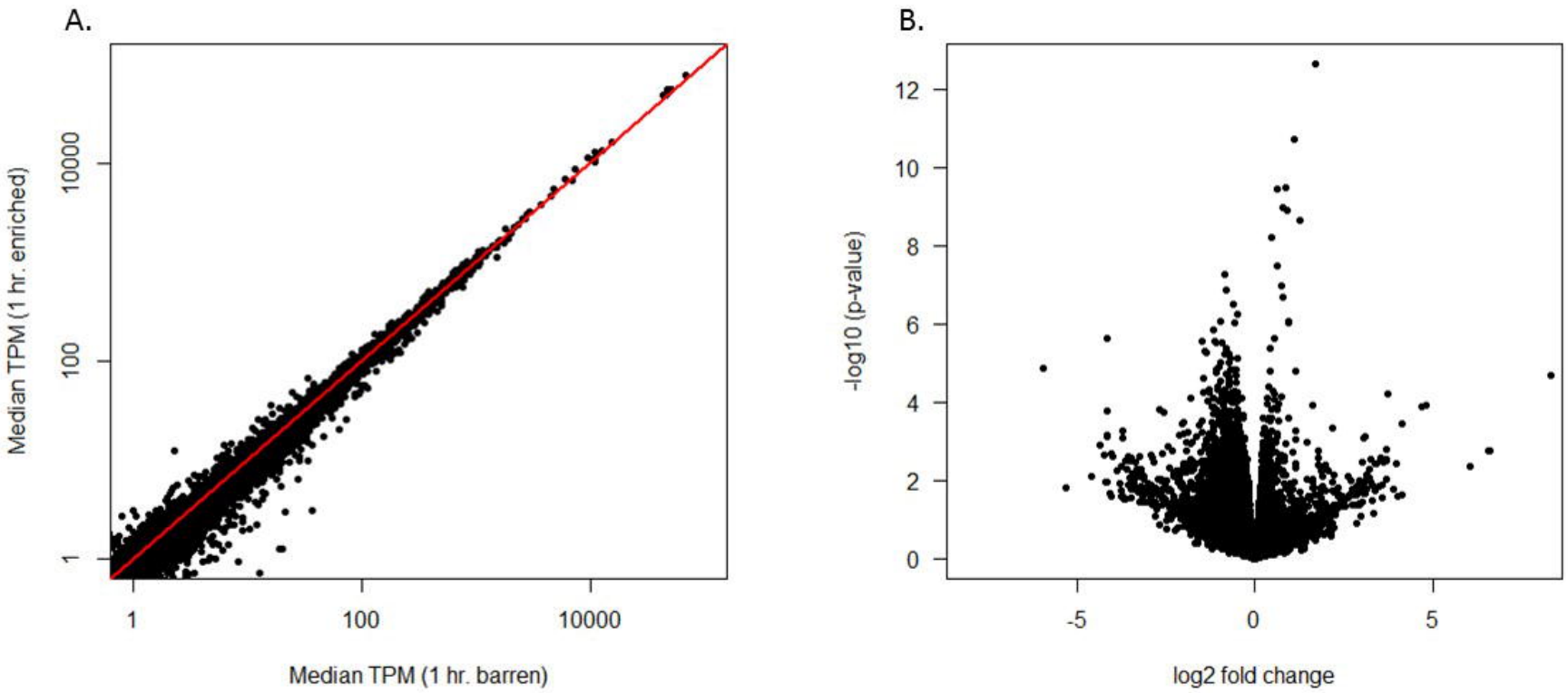
745 **Figure S1**: Cluster graphs from 1 hour Cluster lists found in Table S3 that show clear segregation
746 between EE and B animals. Panel (A) Microglia and connective tissue genes; Panel (B) Immune and
747 connective tissue genes; Panel (C) IEG; Panel (D) All clusters. Each column represents a single animal
748 with B house pigs as columns 1-6 and EE pigs columns 7-12. There is clear segregation in panel D of
749 the EE animals (top clusters) and B animals (bottom clusters)
750
751 **Table S1**: Means and SEM of behaviours at all time-points by treatment.
752
753 **Table S2**: Differentially expressed genes at 1 and 4 hours. With log2 fold change, p value, average
754 counts and average transcripts per million for each treatment group.
755
756 **Table S3**: Genes present in gene-gene cluster analysis.
757

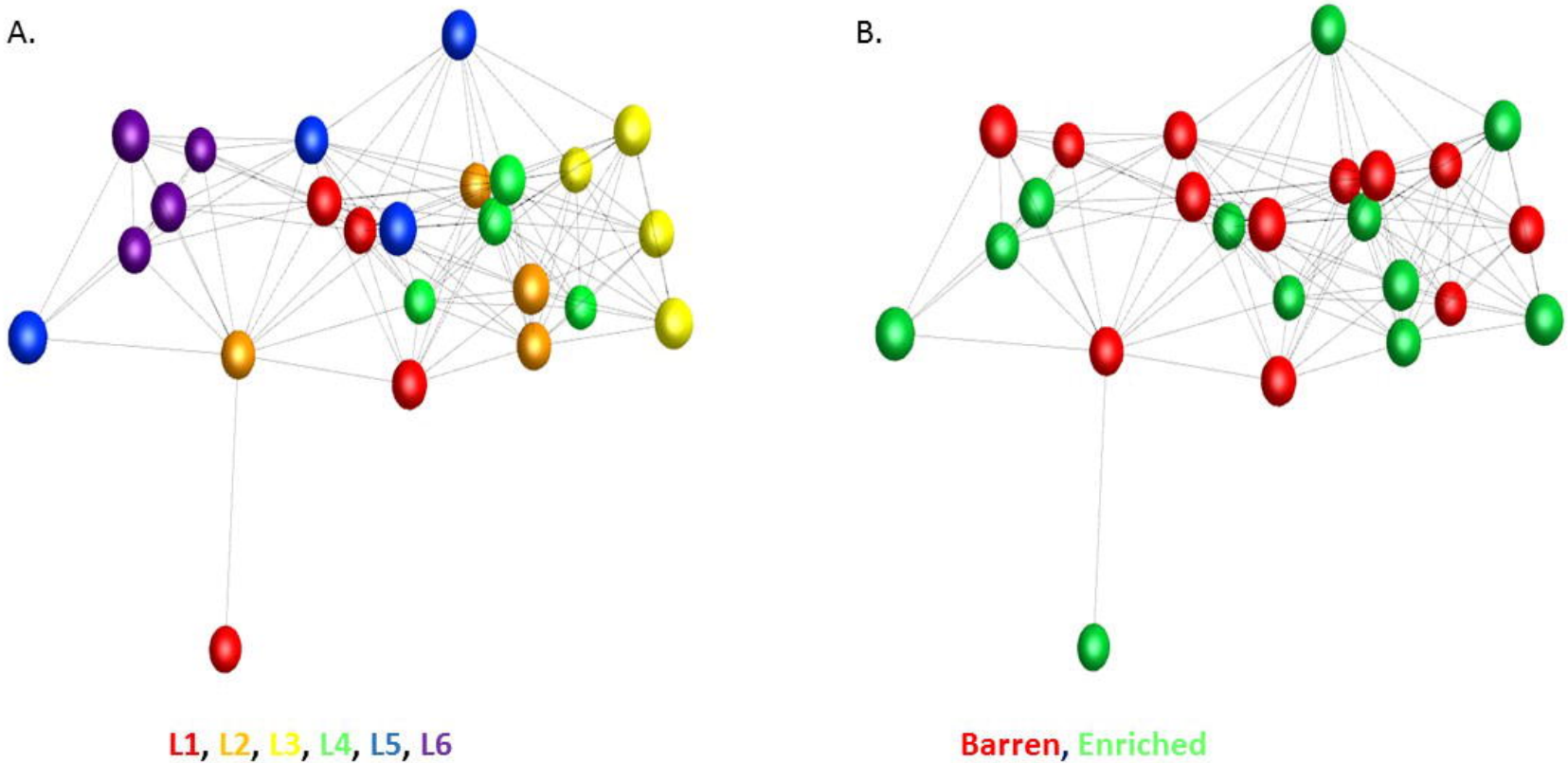
758 **Table S4**: IEG and microglial reference gene list. Reference list used for randomisation tests.

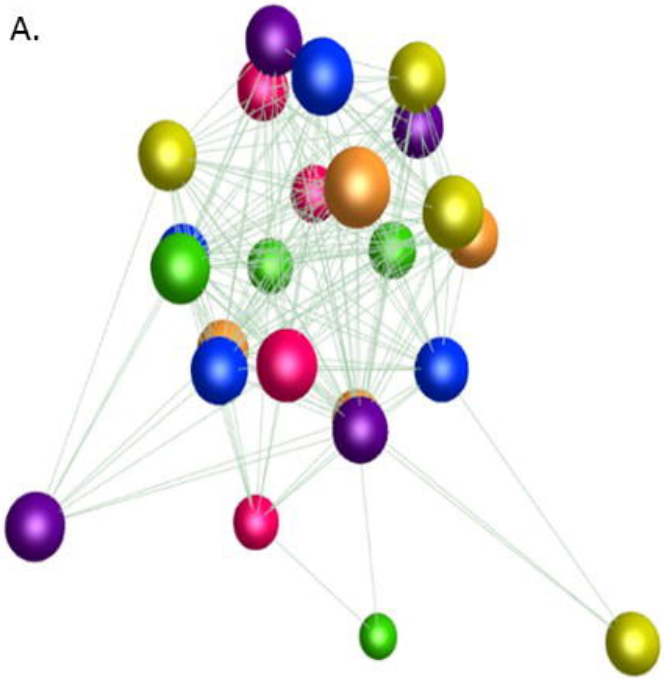
759


L3
L4
L2
L5
L1
L6
L1
L2
L3
L6
L5
L4

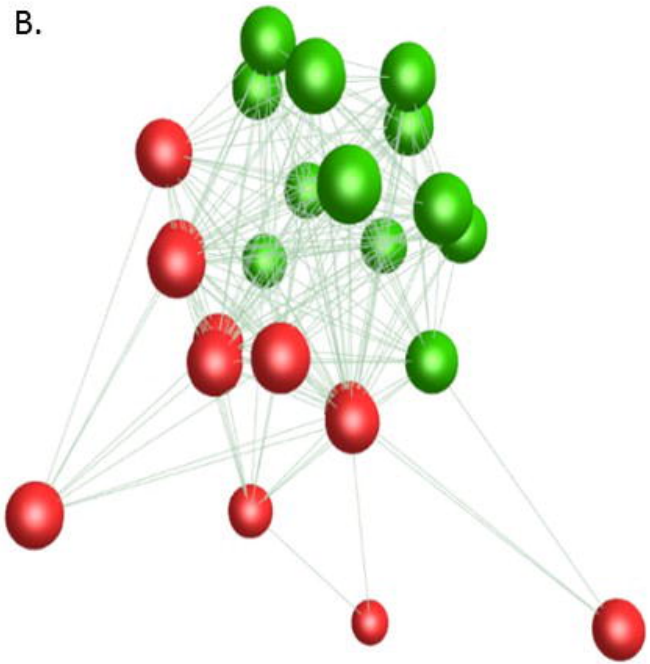

Enriched
Barren
60
50
40
30
20
10
0
% ACTIVE BEHAVIOURS
Pre
1hour post
2 hour post
3 hour post
4 hour post
TIME PERIOD


A.
Median TPM (1 hr. enriched)
10000
100
1
1
100
10000
Median TPM (1 hr. barren)
B.
-log10 (p-value)
12
10
8
6
4
2
0
-5
0
5
log2 fold change


A.
B.
L1, L2, L3, L4, L5, L6
Barren, Enriched



L1, L2, L3, L4, L5, L6



Barren, Enriched