Validation of MicroRNA as Biomarkers of Affective State

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Abstract

Positive affective state is the subjective experience of positive emotions and is linked to well-being and resilience. Animal welfare now advocates for the promotion of positive affective experiences in addition to reducing negative experiences. Positive affective state in animals can be determined through judgement bias but this is a lengthy procedure inefficient for industry. A rapid non-invasive biomarker to assess positive well-being of animals is essential to inform ethical industry practice.

MicroRNA (miRNA) are stable molecules found in most mammalian cell types which post-transcriptionally regulate target genes altering biological mechanisms. MiRNA expression is altered by changes in environment. There has been much research on identifying miRNA as biomarkers for depression, but there is limited knowledge regarding their use as biomarkers of positive affective state. The aim of this study was to identify miRNA which are differentially expressed over time in oppositely valenced affective states created through environmental manipulation.

Housing environment of rats was used to create change in valence of affective state which was validated by judgement bias test. MiRNA extracted from blood and amygdala tissue were analysed for differential expression between housing groups at Day 3 and Day 10.

Metabolic cages created significant negative bias compared to home cages at Day 10 ($p=.024$). Metabolic cage housing associated with negative bias drove miRNA expression changes in blood with multiple miRNA significantly upregulated. Of these, miR-193, miR-142 and miR-326 regulate pathways associated with physiology, immune response, and the dopaminergic system informing mechanisms of well-being with potential as biomarkers.

Introduction

Attitudes to animal welfare are dynamically challenging industry's ability to maintain their social licence to farm livestock. Treatment of animals has progressively become an important issue guiding practice of ethical agriculture and welfare of laboratory animals[1]. An older definition of animal welfare encompasses animals' access to food, water and appropriate housing while being free of injury or disease and enabling innate behaviours[2]. More recently this definition has expanded to include the promotion of positive affective experiences for the animal rather than solely reducing negative states[3]. Resilience[4] and well-being[5] are linked to subjective experiences of positive emotional states like joy and enthusiasm. Depression and anxiety in humans are associated with negative or less positive affective states[6]. Similar phenotypes are found in animals who experience harsh environments[7] and handling techniques[8]. Emotions launch adaptive responses of cognition and action allowing for integrated physiological reactions to the external environment[9]. Activated physiological systems, such as the immune system, have bidirectional association with affective states[10] providing potential avenues to measure positive experiences in animals.

Arousal vs valence of affective state

Perception of external sensory information is processed and bound to physiological reactions[11]. How we sense and comprehend the environment around us is reflected in our emotional response. Emotional arousal is a measure of the intensity of an affective state, observed through behavioural and physiological indices such as levels of glucocorticoids[12]. However, these measures do not determine valence, whether the experience is felt as positive or negative. For example, heart rate may increase when encountering a predator or a sexual partner; situations presumably evoking two oppositely valenced emotions[13]. Although arousal provides some measure of affective state, valence of affective state determines the impact, positive or negative, on the animal with implications for

welfare[13]. Valence of emotion influences bias in cognitive processing including attention, memory and judgement[14].

Judgement bias test

Affective state at any point in time is comprised of trait affect, environment, and previous experience[13]. Affective state influences cognitive processing of information creating bias in decision-making which reflects the underlying affective state whilst cognitive perception of stimuli shapes emotional responses[13]. Bias in cognitive processing is an attested indicator of affective state and emotional valence, and bias in animals is discerned using a judgment bias test[13]. The judgement bias test involves combined facilitation of the animal's sensory, cognitive and movement systems. The animal senses the environment, evaluates stimuli from memory of associations previously made, selects a response and performs an action[13]. The active choice judgement bias test determines relative affective state through the interpretation and subsequent action the animal makes to an ambiguous stimulus[13]. Actions associated with expectation of a positive or more valued event (optimistic choice) in response to an ambiguous stimulus suppose a positive affective state while actions associated with an expectation of a negative event or lower reward (pessimistic choice) suppose a negative affective state[13].

Environment manipulates affective state

There is much evidence that cognitive processing of animals is biased by environmental conditions and more optimistic choices, expecting a positive event, are made when in an enriched environment[15, 16]. Barker et al. showed that the environmental change from housing conditions altered the number of optimistic choices made by rats in a judgement bias test[17]. Housing in metabolic cages induces behavioural changes in rats inferring a negative environment[18]. Male rats housed alone in metabolic cages showed a significant decrease in the number of optimistic decisions made in response to an ambiguous stimulus[17]. Environmental enrichment induced

optimistic cognitive bias in rats when transferred from an unenriched cage suggesting a more positively valenced affective state was created[15].

The role of the Amygdala in affective state

The underlying biological mechanisms of affective states are not yet understood, however certain brain regions have been identified. The amygdala is activated in response to stimuli which elicit emotions, both negative and positive[19]. Activation of the amygdala, part of the emotion regulatory limbic region, has been linked to reward-seeking behaviour[20]. The transcriptome of the amygdala has not been well studied to date and may reveal insights into cellular and molecular processes of affective state.

MiRNA and positive affective state

MiRNA are small non-coding RNA (20–22 nucleotides) which regulate gene expression post-transcriptionally. Whilst miRNA are particularly prevalent in brain sites, they can be detected in components of the blood[21] where they reflect physiological conditions[22]. MiRNA are downregulated or upregulated rapidly in response to changes in environment which alter the affective state of animals[23]. MiRNA preserve their stability in tissue samples and can be readily extracted and evaluated, providing a practical tool for a clinical biomarker[22]. MiRNA have previously been studied for their association with depression and other psychiatric disorders[24]. However, there is limited literature on the association of miRNA with positive affective state.

Two studies have identified miRNA associated with positive affective state. Firstly, Wingo et al, [25] identified a single nucleotide polymorphism (SNP), rs322931, in humans which predicted expression of miR-181 in positive affective state. MiR-181a in the nucleus accumbens of rats influences synaptic plasticity[26] and miR-181a/b helps regulate T-cell receptor sensitivity in mice[27] thus connecting miRNA expression within reward centres of the brain and the immune system to

positive affective state. The second study identified miR-132 expression in the basolateral amygdala (BLA) in mice as having a role in learned safety, through modulating expression of genes which determine neural excitability and synaptic plasticity[28]. Learned safety relates to positive affective state and a reduction of depression-like behaviour in mice through identifying experiences of security[29].

Objective

The judgement bias test has been validated as a tool for measuring valence of affective state in animals[30]. However, training in the judgement bias paradigm takes days to complete rendering it impractical for use in the agricultural field. Combinations of miRNA associated with positive affective state may be useful as biomarkers facilitating an objective welfare assessment tool via a quick, relatively non-invasive sampling method. The objective of this project was to firstly create two differently valenced affective states in rats through environmental manipulation, determined through a judgement bias test. Rats are used as a representative species with the assumption that findings will translate across species. The second aim was to explore differential expression of miRNA between the validated affective states between two timepoints and to investigate correlation of expression between amygdala and blood tissue. The hypothesis of my study is that environmental manipulation of affective state, as determined through a judgement bias test, will induce temporally relative differential expression of miRNA which will be correlated between blood and brain tissue providing a potential biomarker of affective state.

Materials and Methods

Animals

The study was granted ethics approval (#33110(v2)) by the University of Adelaide Animal Ethics Committee in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes[31] and the *Animal Welfare Act* (SA)[32].

Animals were sourced from a barrier maintained, specific pathogen free production facility (University of Adelaide, Laboratory Animal Services, Adelaide, Australia). Rats were male (n=42) Sprague-Dawley outbred strain (Hsd:SD) which arrived at 4-weeks of age, at different intervals, and were initially housed in groups of 6 into Individual Ventilated Cages (IVC). Animals were habituated in the IVC home cage (Tecniplast Double-Decker IVCs, 4620 x 4030mm) for a minimum of 5 days. Animals had ad libitum access to a food hopper containing dry pellets (Teklad Global Soy Protein-Free Extruded Rodent Diet), and two water drippers containing reverse osmosis water during their training and experimental phases. Animal weights were recorded daily.

Experimental design

The experimental design to determine valence of affective state was based on a shortened protocol for the judgement bias test proposed by Brydges & Hall[30]. The procedure is explained in brief in Table 1 with testing apparatus shown in figure 1. Training involved associating a more highly valued reward, chocolate, with fine grade sandpaper and the lesser reward, a cheerio, with coarse grade sandpaper. During the judgement bias test an intermediate grade sandpaper was used as the ambiguous stimulus and responses documented.

Table 1. Schedule for shortened judgement bias paradigm

	Judgement bias training schedule
Habituation	Animal placed in apparatus for 5 mins, no rewards, no tactile stimuli
Phase 1 training	Free access to both compartments which contain a cheerio or chocolate
Phase 2 training	Exploring tactile association to rewards and encouraging foraging
Phase 3 training	Reinforcing association of a tactile stimulus to a reward
Phase 4 probe trial	Baseline trial for all animals – randomized ambiguous tactile stimulus
Separation	Animals randomly moved to treatment cages
Phase 4 probe trial	Randomized trials with ambiguous tactile stimulus at Days 3 and 10
Primary outcomes to measure	Optimistic, pessimistic or No choice Latency to make a choice, max 5 mins

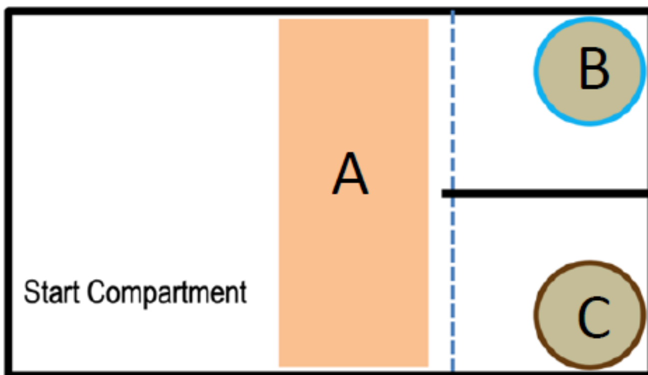

Figure 1: Schematic of testing apparatus utilised in judgement bias test. (A) sandpaper as stimulus, (B) & (C) reward bowls. (Diagram reproduced from Cutler, 2019[33]unpublished).

Animals were tested for baseline data where the ambiguous stimulus was first encountered. Animals were then randomly separated into two housing conditions: isolated in a metabolic cage with metal grid floor and no shelter (220 mm in diameter × 120 mm tall) or remaining in IVC. Animals were kept in housing conditions for 3 day or 10 day durations (figure 2). At the end of

these timepoints animals were again tested in the judgement bias paradigm, followed by humane euthanisation with carbon dioxide. Brain and blood tissue were collected at this point.

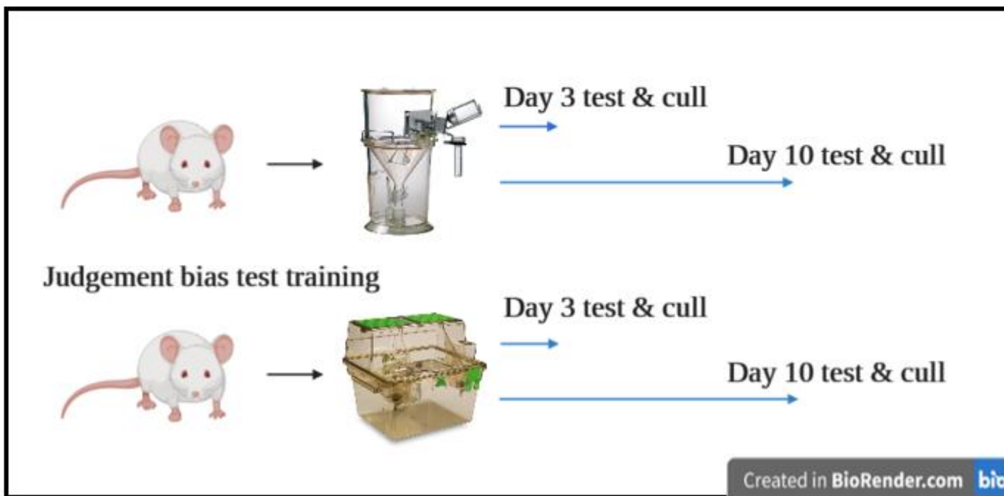

Figure 2. Rats were randomly allocated between metabolic cage and IVC housing and further distributed into temporal groups (3 or 10 days). Cognitive biases validated affective state through judgement bias test (n=12 per group with n=6 for Day 10 IVC group).

Tissue collection and storage

Blood was taken by cardiac puncture from rats immediately following euthanasia, put into RNAprotect® Animal Blood Tubes (QIAGEN) as per manufacturer instructions and stored at -80°C. Rat brains were extracted, cut with scalpel into forebrain, middle and hindbrain using a brain matrix as guide, then stored separately in RNAlater™ Stabilisation Solution (ThermoFisher) at -80°C. Judgement bias testing and tissue collection undertaken by Cutler[33] in 2019.

Tissue sample size

A sample size of 5 was used per group for miRNA extraction. Tissue was randomly assigned from each housing condition (metabolic, IVC), and each timepoint (Day3, Day 10) using the free online website https://miniwebtool.com/random-picker/. This gave a total of 20 samples of brain tissue and 20 samples of blood tissue.

Microdissection

The frozen brain tissue containing the amygdala (n=20) were thawed on ice. Rat brain identity numbers were randomised as to which side, left or right amygdala, would be utilised: 11 samples of left and 9 samples of right amygdala. The 2004 Allen Institute for Brain Science, Allen Mouse Brain Atlas[34] was used as a guide with a dissecting microscope utilised to extract the amygdala. Amygdala samples were separately homogenized using a Kimble ® PELLET PESTLE® Cordless Motor and added to 1-Thioglycerol/Homogenization solution from the Maxwell® 16 miRNA Tissue Kit (Promega) according to manufacturer's protocol and stored overnight at -80°C.

MiRNA Extraction

Amygdala miRNA was extracted over two days:10 samples per day. The frozen homogenised amygdala were thawed on ice. MiRNA extraction from the amygdala was undertaken according to manufacturer's protocol using the Maxwell® 16 miRNA Tissue Kit (Promega) with the Maxwell® 16 Instrument (Cat.# AS2000) updated to firmware version 4.98.

MiRNA extraction of blood tissue was undertaken using the Qiagen RNeasy® Protect Animal Blood Kit according to manufacturer's protocol. 20µl of elution buffer was added per sample to obtain final miRNA elute.

Quantification of miRNA was verified using the BioTek SynergyMX multi-mode microplate reader. The miRNA elutes were then stored at -80°C.

The required concentration of miRNA of 500ng/8ul per sample was prepared by adding nuclease free water and delivered to the Australian Genome Research Facility (ARGF) Cancer Genomics Facility, Centre for Cancer Biology, Adelaide, Australia for gene expression microarray analysis.

MiRNA microarray analysis

The miRNA expression profiles were analysed by ARGF Cancer Genomics Facility, Adelaide, Australia using the Affymetrix® GeneChip miRNA 4.0 array for multi species. MiRNA from brain

and blood tissue was biotin-labelled using the FlashTag™ Biotin HSR RNA Labeling Kit for GeneChip miRNA Arrays (Thermo Fisher Scientific) after Poly (A) tailing. Labelled miRNA samples were hybridised to GeneChip miRNA v4.0 arrays and incubated in a GeneChip® Hybridization Oven 645 for 16 hours at 48°C. Chips were washed and stained using a Affymetrix® Genechip Fluidics Station 450 and then scanned with an Affymetrix® GCS 3000 7G scanner. CEL files of signal values were generated using Affymetrix® GeneChip™ Command Console software.

Statistical analysis

Choice response of rats to ambiguous stimuli: optimistic, pessimistic and No choice, were analysed using Pearson's Chi-square test. Latency data was analysed using the Mann-Whitney test as data was found nonparametric by the Shapiro-Wilk test. Graph Pad Prism 8 was used to analyse choice and latency data. Using box-plots to identify upper and lower limits of 2 standard deviations (SD), outliers were removed from the baseline and Day 10 latency data before analysis[35]. Due to low number and differing sample size for the Day 10 latency data, a corrected effect size was calculated using Hedges' g. Weight gain was analysed as percentage of baseline weight using Mann-Whitney test.

The raw data CEL files were imported into the Transcriptome Analysis Console (TAC) 4.0 software downloaded from the ThermoFischer website. Data was normalised and analysed for rat by selecting miRNA level RMA+DABG-Rat analysis. The analyses conducted in TAC were one-way ANOVA with Bayesian correction. Probe sets were considered as biologically significant if the fold changes (FC) were > 2 with false discovery rate (FDR) < 0.1.

Results

Judgement bias test choice responses

There was a significant difference in the percentage of optimistic choices made at Day 10 (χ^2 (2, n=6-12) = 7.4, p= .024) between IVC and metabolic cage groups (figure.3). There was no significant difference in judgment bias choice response between grouped animals at baseline before allocation to the two housing conditions (χ^2 (2, n=42) = 0.17, p= .92). Animals at day 3 showed no significant difference in judgment bias choice response between IVC and metabolic cage groups (χ^2 (2, n=12-12) = 5.6, p= .061).

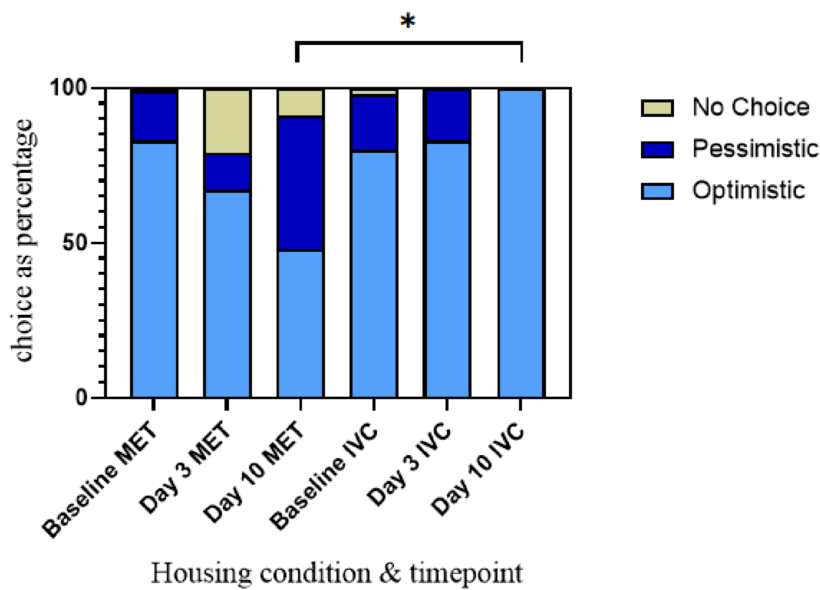

Figure 3. A higher percentage of optimistic choice (per group) was made in the IVC compared to metabolic cages at Day 10. Asterisk denotes significance (p=.024). Choice data analysed using a Chi-square test. (MET: metabolic)

Judgement bias test latency

No significance was found in latency of decision time between grouped animals at baseline before allocation to housing conditions after outliers over 2 SD were removed (p=.063). At day 3 no significance was found in latency of decision time between animals in metabolic cage compared to animals in IVC group (p=.074). After removing an outlier, a large effect size was found in latency between rats (n=8) housed in metabolic cages (M=41.4, SD=39) compared to rats (n=3) in IVC (M=5.7, SD=0.29, 95% CI[-16, 87], Hedges' g= 1.1. Reduced data samples due to technical failure. Metabolic cage groups showed increased inter-individual variability in latency over time (figure 4.).

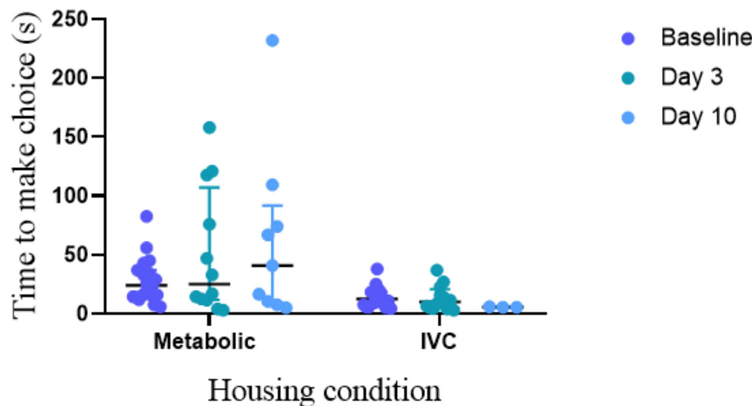

Figure 4. No significance found for latency in decision time between housing conditions. However, variability between individuals increased over time within the metabolic cage group. Data presented as median with IQR. (Sample sizes varied due to technical failure).

Weight gain

The difference in median percentage weight gain of animals significantly increased between housing conditions at Day 10 (p=.033). Inter-individual variability in weight gain increased in animals from metabolic cages becoming more pronouced at Day 10 (figure 5).

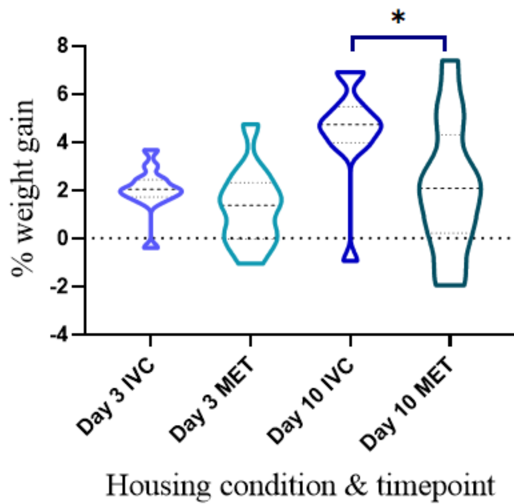

Figure 5. Inter-individual varaition in weight gain was more pronounced in metabolic cage group (MET). By Day 10 the gap in percentage weight gain of the groups showed significance (p=.033) between housing conditions.

MiRNA expression

Blood of rats from metabolic cage group showed expression of 10 miRNA probes were significantly upregulated (FDR<0.1) in Day 10 animals compared to Day 3 animals (Table 2). These 10 miRNA probes related to seven miRNA families: miR-25, miR-142, miR-193, miR-326, miR-328, miR-331 and let-7 with one unknown association (Table 3). Expression of these blood miRNA show inter-individual variation from Day 3 and Day 10 animals in metabolic cages (figure 6). The expression of several blood miRNA reveal greater sensitivity to housing condition and duration as shown in figure 7. In addition, blood levels of rno-miR-190a-3p in the metabolic cage group, although not significant (FDR=0.38), showed a downregulation with FC=16, p=.02 in Day 10 animals compared to Day 3 animals.

Table 2. Blood miRNA differentially expressed between housing conditions and timepoints.

Blood samples	Comparing	MiRNA differential expression FC>2, FDR<0.1
Metabolic cage	Day 3/Day 10	10 miRNA
IVC	Day 3/Day 10	Nil
Day 3	Metabolic cage/IVC	Nil
Day 10	Metabolic cage/IVC	Nil

(FC: fold change; FDR: False discovery rate)

Table 3. Upregulation of blood miRNA from metabolic cage animals at Day 10 compared to Day 3.

miR family	**miRbase iD**	**FC**	**p-value**	**FDR**
miR-326	rno-miR-326-5p	5.3	0.0003	0.078
miR-331	rno-miR-331-3p	4.5	0.0001	0.068
let-7	rno-let-7d-3p	3.5	0.0003	0.078
miR-193	rno-miR-193-3p	3.1	0.0005	0.086
miR-328	rno-miR-328a-3p	2.7	8.40E-05	0.068
miR-326	rno-miR-326-3p	2.5	0.001	0.098
miR-25	rno-miR-92b-3p	2.3	0.0005	0.086
let-7	rno-let-7a-5p	2.1	0.0007	0.086
miR-142	rno-miR-142-5p	2.0	0.0007	0.086

(FC: fold change; FDR: False discovery rate)

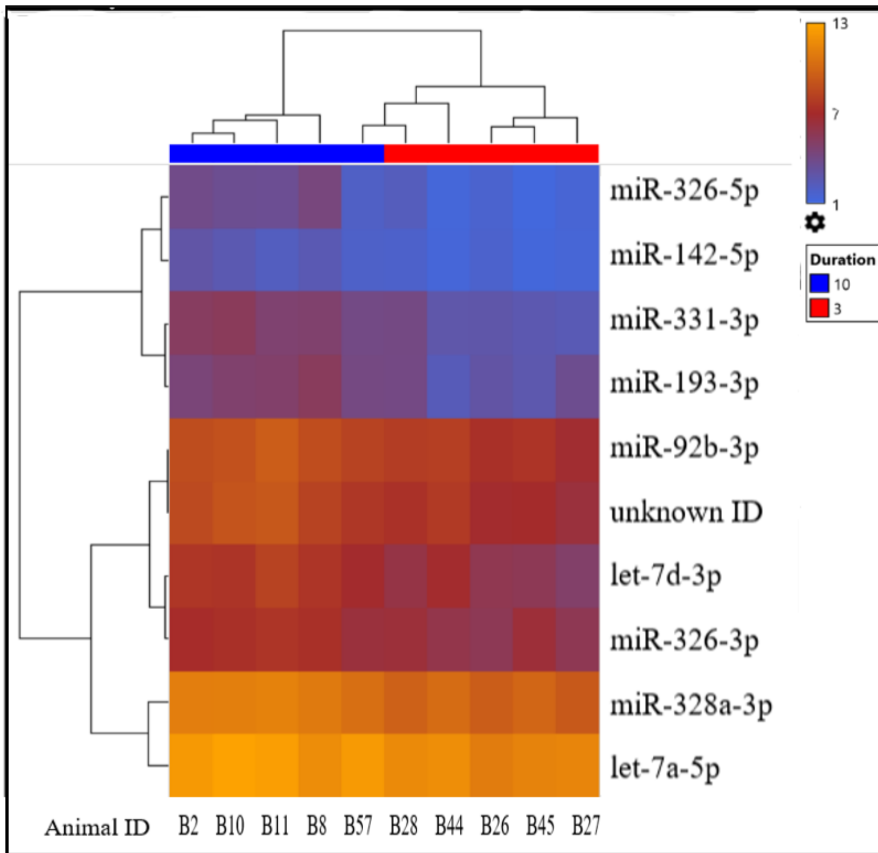

Figure 6. Inter-individual variation shown in heat map. Blood miRNA expression of rats from metabolic cages were differentially expressed between Day 3 and Day 10 animals. Day 3 animals depicted under red bar. Expression intensities in log2. Heat map generated through TAC software.

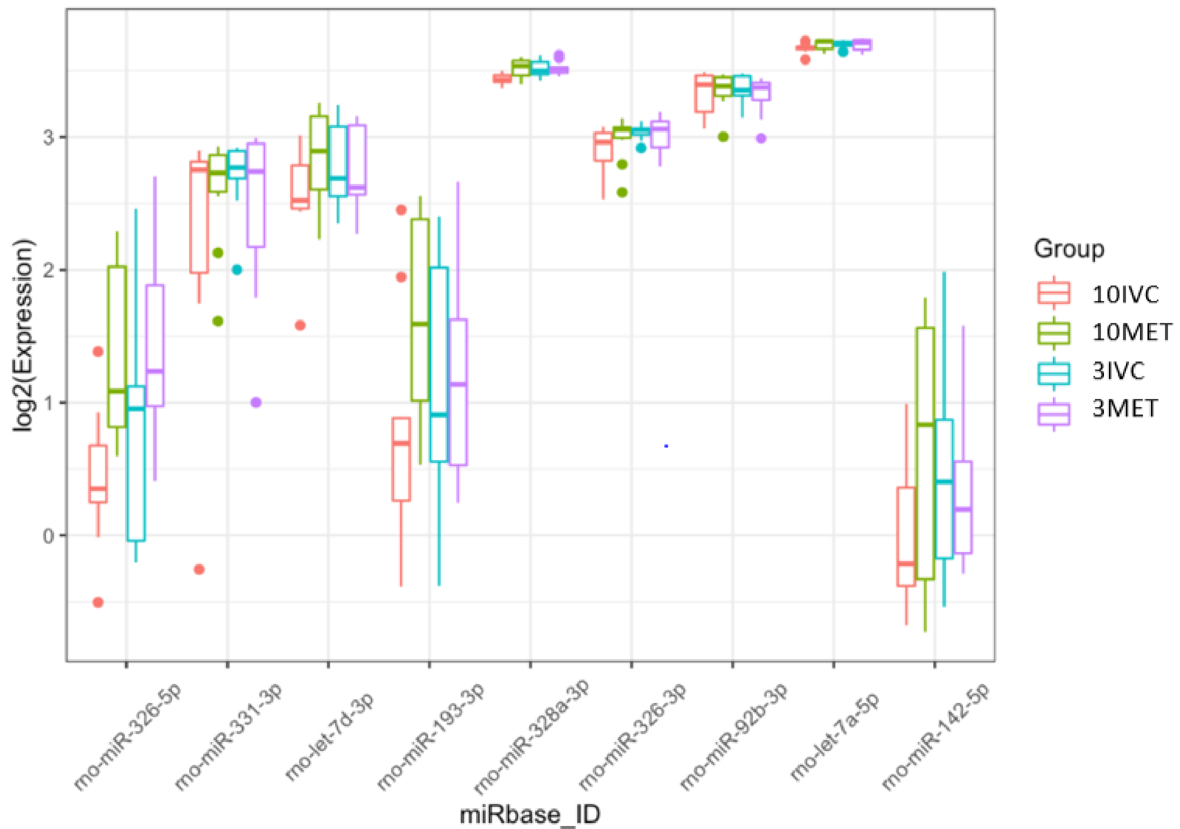

Figure 7. Sensitivity to changes in housing conditions and duration is greater for several miRNA. Blood miRNA expression shows large variability between and within treatment groups. Graph generated through R by Kelly Ren. (10IVC, 10MET: 10 day, 3IVC, 3MET: 3 day)

A search of PubMed literature starting with the last 5 years revealed expression of miR-193-3p, miR-142-5p and miR-326-5p are involved with regulation of the dopaminergic system. These three, in addition to miR-190a-3p, are also potentially facilitating stress response pathways relating to physiology and the immune system (Table 4).

Table 4. Metabolic cage driven blood miRNA are associated with functions relating to stress.

MiRNA	Expression	Associated biological functions	Targets	References
miR-193-3p	↑	dysfunction of tyrosine hydroxylase, insufficiency of dopamine synthesis	ALDH2	36
miR-142-5p	↑	dopaminergic neurotransmission	SIRT1	37
	↑	impairment of spatial memory, emotion-related behaviour	Npas4	38
	↑	critical regulator of dendritic cell homeostasis		39
	↑	immunometabolic regulator of intracellular cAMP	regulatory T cells	40
	↑	pathogenesis of autoimmune neuroinflammation	Tcell differentiation	41
		cardiac muscle cell survival	cytokine receptor gp130	42
		angiogenesis	vessel tone	43
	↑	apoptosis in human macrophages with link to atherosclerosis	TGF-β2	44
	↑	induced in fever	IL-6, IL6ST, TLR2, PGE2, TNF	45
miR-326-5p	↑	increase autophagy of dopaminergic neurons via JNK signalling pathway	XBP1	46
	↑	reduced dopamine receptor D2 mRNA and receptor synthesis		47
	↑	promote T helper 17 (TH-17) cell differentiation	Ets-1	48
	↑	immune response, down-regulation of pro-inflammatory cytokines	TLR4	49
	↑	endoplasmic reticulum stress, mitochondrial fission, apoptosis	PKM2	50
miR-190a-3p	↓	regulates insulin sensitivity	IGF1	51, 52

There was no differential expression of miRNA identified through TAC software in the amygdala between housing conditions or between timepoints (Table 5). Correlation of miRNA differential expression between the amygdala and blood tissue could not be identified.

Table 5. Amygdala miRNA differentially expressed between housing conditions and timepoints.

Amygdala samples	Comparing	MiRNA differential expression FC>2, FDR<0.1
Metabolic cage	Day 3/Day 10	Nil
IVC	Day 3/Day 10	Nil
Day 3	Metabolic cage/IVC	Nil
Day 10	Metabolic cage/IVC	Nil

(FC: fold change; FDR: False discovery rate)

Discussion

The negative environment of the metabolic cage[18] by Day 10 induced a difference in the informational processing of rats demonstrating negative bias as determined through the active choice judgement bias test. This supposes the affective state of these rats was negatively valenced. In addition, rats in metabolic cages gained less weight. The stress response induced from a negative environment activates the hypothalamic–pituitary–adrenal (HPA) axis releasing stress hormones which mobilise energy from adipose tissue and sustain the immune system[53]. Rats in the metabolic cages may have undergone physiological mechanisms converting lipid storage to energy to maintain the stress response, hence gaining less weight. Energy demand from the brain increases during times of stress so while metabolic caged rats are limited in movement, the cognitive processing involved may be energy intensive[54].

The amygdala is crucial for stimulus–reward learning[55] and processing of external stimuli[19]. The amygdala extracts information with biological relevance from the environment[19] and moulds

ensuing behavioural responses[56]. Although my study found no difference in amygdala miRNA expression between treatment groups, Haramati et al.[57] found differential expression of miRNA in the amygdala of adult mice exposed to acute stress compared to controls. The region of the amygdala includes multiple nuclei with functional segregation. Evidence has shown the BLA structurally connects with areas of sensory information, whereas the central nucleus of the amygdala signals the endocrine, autonomic, and somatomotor pathways[58]. This variation in function of the amygdala nuclei may have confounded results in discerning patterns of miRNA expression; miRNA have cell type and tissue specificity[59]

In parallel with behavioural changes at Day 10, blood expression of multiple miRNA from metabolic caged animals were upregulated in day 10 animals compared to day 3 animals suggesting the negative experience had temporally changed genetic functioning of systems manipulating cognitive processing of the animals. These miRNA with altered expression, through the target genes they post-transcriptionally regulate, may reveal pathways linked to affective state.

Exosome mediated transport of miRNA suggest a mechanism of cell-to-cell communication between the brain and the periphery[24]. Exosomes are membrane vesicles (50–90 nm) of endocytic origin and were found to contain miRNA expressed at higher levels than in cells[60]. Exosomes have potential as mechanisms of communication through the systemic circulation in a way resembling hormones, allowing miRNA regulatory communication between mammalian cells; exosomes contain molecular components linked to the specificity of their original cells[61]. There is potential that the miRNA found upregulated in the blood of animals in the metabolic cages comprise a miRNA signature, communicating neurophysiological activity influencing behaviour. Identifying circulating exosomal miRNA regulating gene expression of mechanisms associated with brain regions involved in positive affective state could progress research towards a biomarker for animal welfare.

The inter-individual variability within groups (figure 6) shows individuals have their own miRNA expression levels. This variability increases (figure 7) with several blood miRNA expression showing greater reactivity to different conditions. Increased variability in blood miRNA expression in response to altered conditions occurred with miR-326-5p, miR-193-3p and miR-142-5p (figure7). Patterns of expression for particularly sensitive miRNA could predict resilience or well-being in an individual animal; combinations of these could identify a miRNA signature, suggesting epigenetic regulation as an individual's adaptive response to environment.

Dysfunction of the dopaminergic system has been linked to the stress response and the HPA axis, limiting the ability to select and process significant environmental stimuli[62]; dopamine neurotransmission is connected to motivated action towards reward[62]. A miR-142-5p target gene is involved with stress-induced brain dysfunction and decreases in brain-derived neurotrophic factor (BDNF)[63] and miR-326 overexpression has been linked to reduced dopamine connectivity. Studies reveal circulating miR-193 affect schizophrenia[64] and Parkinson's disease[65], and may regulate the amyloid precursor protein in Alzheimer's disease[66] .

Dysregulation of the immune system has been linked to pathophysiology. MiR-326 is associated with TH-17cells which secrete interleukin-17 (IL-17), implicated in neurological diseases associated with cognitive impairment and IL-17 was shown to disrupt the blood brain barrier integrity[67]. MiR-326 are highly expressed in neurons[68] and through overexpression may form a pathway where the brain regulates the stress adaptation response. MiR-142 inhibits the cytokine signalling mechanism[42], is induced in fever[45], and is a potential biomarker for acute myocardial infarction[69].

Overexpression of miR-326 stresses physiological pathways impairing glycolysis with implications for energy availability[50]. The downregulated miR-190a found in my study targets insulin-like

growth factor 1(IGF1); higher levels of IGF1 are related to depression and anxiety and could be a protective mechanism against impaired neurogenesis resulting from a stress response[70].

Limitations of the study

Chen et al.[71] suggests that miRNA expressed in the medial pre-frontal cortex (mPFC) rather than the BLA are important indicators of resilience or vulnerability to stress. The pre-frontal cortex (PFC) is the site of executive function, influencing behavioural impacts of amygdala activation[72]. PFC neurons combine associations between previous experience, voluntary actions, and reward value[73]. The PFC may be involved in a mechanism inhibiting dopamine output in the nucleus accumbens thereby affecting goal-directed behaviour and amygdala-mediated stimulus-reward association[72]. MiRNA from the PFC may show greater differential expression in accordance with differing behaviours that arise from alteration in information processing. Although the amygdala provides emotional processing of perceptions from the external sensory environment, the PFC, downstream of the amygdala, is a potential region of miRNA expression change; regulating genes to enable functions evolutionarily preserved for an adaptive response.

A difference in judgement bias was identified at Day 10 between the two housing conditions, however no difference was found in MiRNA expression. Possibly, miRNA expression is too specific to the individual to act as a biomarker of affective state. However, including baseline data of miRNA expression or other timepoints may have revealed differences. MiRNA respond rapidly to environment; a study by Lecchi et al.[23] detected changes in miRNA expression in turkeys after two hours of environmental stress. The judgement bias test itself may provide a novel environment for rats housed in the metabolic cage but increase stress for those taken from their home cage to a new environment[74], reducing any differences in miRNA expression immediately before euthanasia. Another concern is the use of the IVC as the alternative to the metabolic cage which may have created an insufficiently positive environment for distinguishing mechanisms regulated by miRNA.

The sample size may have been too small and inter-individual innate differences of rats, such as dominance traits, may have confounded results. A non-dominant rat may experience relief housed in a metabolic cage away from dominant rats. Also, animals had ad libitum access to food and if recently satiated would have less appetitive motivation to seek out food rewards in the test, resulting in increased latency or 'No choice' responses recorded in the allocated time.

Future directions

Multiple miRNA in blood upregulated temporally in response to metabolic cage housing. Biological mechanisms indicated by target genes of these miRNA included pathways of physiology, the immune system and the dopaminergic system which have identified associations with stress response. Further study could quantify these identified miRNA and target genes by using real-time polymerase chain reaction analysis and western blot in a replicated experiment adding measures of stress response such as glucocorticoid and BDNF.

Conclusion

Resilience may come from a genetic predisposition or a learned behaviour, halting progression of stress responses (an initial adaptive behaviour) from entering a negative downward spiral. Although no correlation between amygdala and blood miRNA was identified in this study at the chosen timepoints, the blood miRNA found to be differentially expressed between timepoints in the metabolic cage group may identify physiological markers relating to resilience to an external environmental stressor. These circulating miRNA may be initial candidates for exploration of a biomarker to determine resilience and positive affective state, providing a tool for animal welfare.

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