The effects of graded levels of calorie restriction XV: phase space attractors reveal distinct behavioral phenotypes

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Abstract

Calorie restriction (CR) has a positive impact on health and lifespan. Previous work however does not reveal the whole underlying mechanism of bahavioral phenotypes under CR. We propose a new approach based on phase space reconstruction (PSR) to analyze the bahavioral responses of mice to graded CR. This involved reconstructing high-dimensional attractors which topologically represent the intrinsic dynamics of mice based on low-dimensional time series of movement counts observed during the 90 day time course of restriction. PSR together with correlation dimensions (CD), Kolmogorov entropy (KE) and multifractal spectra builds a map from internal attractors to the phenotype of mice and reveals the mice with increasing CR levels undergo significant changes from a normal to a new state. Features of the attractors (CD and KE) were significantly associated with gene expression profiles in the hypothalamus of the same individuals.

Keywords: calorie restriction; phase space reconstruction; attractors; gene expression

1. Introduction

Calorie restriction (CR) is one of the few environmental perturbations that has a positive impact on health and lifespan [1-4]. Despite its discovery 100 years ago (in 1918), and intense modern research activity, the mechanisms underlying its effects remain unclear and disputed. Graded levels of CR lead to graded responses in life span [5-6] and thus the factors that relate to the graded levels of CR are of particular interest [7]. During restriction, mice and other species, consistently alter their behavior, body temperature and body weight [8-10]. We have previously studied the responses of C57BL/6 mice to graded levels of CR, including recording their body composition [11], hormonal status [12] multi-tissue transcripomic profiles [13-16], plasma and multi-tissue metabolomics [17-18] body temperature [10] and physical activity

patterns [19]. These latter two bahavioral components have been monitored every minute over 3 months of restriction using a remote logging device that interrogates a passive implanted transmitter (eMitter: vital view US). These data provide a window to investigate the dynamic responses of mice to the graded levels of CR [11-13, 20-21].

Previous work in these mice has explored some aspects of the dynamic changes that occur under graded CR. For example, Mitchell et al [11] investigated changes in body weight over 90 days of restriction using nonlinear regression analysis and one-way ANOVA. They found that the body mass dynamically changed over the first 30 days under CR but thereafter it stabilized. The time to reach stability was unrelated to the level of restriction. They also studied how the body temperature of the mice responded to graded CR and protein restriction (PR) over 3 months [10]. They showed that during the first 30-35 days, there was a dynamic change in daily average body temperature and then it stabilized until day 70, followed by a further decline. Taking a different approach Lusseau et al [21] used a hidden Markov model to analyse the changes in bahavioral phenotypes related to different levels of CR. They showed for the first time that mice under CR changed the characteristics of their activity states, rather than their activities.

Although some existing work has used time domain methods, such as statistical methods or hidden Markov models to study mice under graded CR, and these approaches can reveal some of the features of the dynamic responses to CR, they do not reveal the whole underlying mechanism of bahavioral phenotypes. In this paper, we develop a new approach based on phase space reconstruction (PSR), or embedding theorem [22-23], to analyze the bahavioral responses to graded levels of CR. We show that the time series of movement counts obtained for mice in this experiment are chaotic, and thus they are appropriate to be analyzed using PSR. PSR can generate the attractor of a system, a subset of the system's phase space, which may be considered as a geometric object to which all the system's trajectories converge. In the mouse

movement situation, PSR generates the attractor of one mouse, which contains all the asymptotic states of the mouse's trajectories. In another word, PSR generates the attractor of one mouse which is topologically equivalent to the actual bahavior of the mouse. An attractor can be further described by its correlation dimension (CD), Kolmogorov entropy (KE) and size. CD characterises the geometric complexity of the attractor of a mouse. A bigger CD means a more complex geometry of an attractor. KE quantifies how complex a chaotic (mouse) system is. The bigger a KE is, the more chaotic the system is. With PSR, we can both quantitatively and topologically have a global view on the dynamics of a system, which is described by the attractor of the system. PSR has been widely used in many areas; see, for example [24-27] for the applications to medical science and ecological science.

In our experiment, we divide the mice into six groups, i.e, 24AL, 12AL, 10CR, 20CR, 30CR and 40CR. 24AL means the mice were fed completely ad libitum without restriction for 24 hours per day; 12AL refers to ad libitum feeding for 12h per day during darkness. 10CR, 20CR, 30CR and 40CR refer to caloric restriction by 10%, 20%, 30% and 40%, respectively, relative to the baseline intake of the same individual mouse. We illustrate our framework including our main idea in Supplementary eFigure 1. Traditionally, in the time domain, the time series of movement counts of mice under different CR levels show different behavior, and the clustering of time series of movement counts simply classifies mice under different CR into different groups. However, while we can superficially see the different effects due to distinct CR, we do not know how the underlying mechanism of bahavioral phenotypes of mice evolves or changes due to exposure to the distinct CR levels. Moreover, the analysis in the time domain may not be accurate due to limited observations. Therefore, we reconstructed a high-dimensional time series based on low-dimensional time series of movement counts observed during the 90 day time course and further obtained the attractors for ten periods, topologically equivalent to real attractors that are not observed in real mice, by evenly dividing the whole 90

day time course of calorie restriction into ten nine-day periods, which represent the intrinsic dynamics. We show that the mice under distinct CR levels undergo significant changes of their underlying mechanism reflected by their attractors' characteristics such as CD, KE and size. It is the change of the underlying mechanism that results in the change of phenotypes under distinct CR levels. It has also been found that the clusters characterized by the attractors are consistent with the classification of mice given by the hierarchical clustering method in the time domain. We also perform multifractal spectrum analysis on each mouse at distinct CR levels, which further validates the conclusions of the PSR.

In particular, we build a map from the intrinsic attractors to the bahavioral phenotypes under distinct CR levels. This map not only explains and characterizes the phenotypes of mice but also predicts their dynamic behaviors due to the asymptotic states of the respective attractor for a biological system. Because we have extensively phenotyped the same individual mice by constructing the phase space attractor, we further leveraged this phenotype to address the relationship between characteristics of the attractors (CD and KE) and gene expression in the hypothalamus, based on the measured RNA-Seq data [13], which provides a unique biological insight into the dynamics of mice at various CR levels.

2. Results

2.1. Three patterns revealed by hierarchical clustering

Figure 1(a) shows the average movement counts of each of six groups of mice with time. These average time series reveal differences in behavior among these groups. We further used hierarchical clustering to group mice together with similar patterns of response (see Figure 1(b)). This classified the mice into three categories. That is, the blue category consists of all the mice from the 24AL and 12AL groups (i.e., mice numbered as 1-16), the green category included mice mainly from 10CR and 20CR groups with one exception (i.e., the mouse numbered 38 from 30CR), and the red category included mice mainly from 30CR and 40CR with three

exceptions (i.e., the mice numbered 18 from 10CR and 27 and 32 from 20CR).

Both the time series and the hierarchical clustering results reveal different phenotypic behaviors under different levels of CR. However, we do not know how the underlying mechanism of bahavioral phenotypes of mice evolves or changes due to the distinct CR levels. To investigate how different levels of CR make the underlying bahavioral mechanism change, we took advantage of PSR to gain more insight into the impacts of CR on the mice by characterising the underlying attractor structure and how this varies over time on restriction and at different levels of restriction.

2.2. Distinct CR levels exhibit different shapes of attractor with PSR

We had six groups of mice exposed to different levels of restriction/food access. Before reconstructing the phase space, we first pre-processed the data by eliminating outliers (see Supplementary Methods for the detailed method) and removing noises with wavelet domain denoising.

For each group, we computed the average movement counts of all the mice, thus producing a new time series of movement counts for each group. This was to understand the collective underlying behavior pattern of each group of mice. To observe the changes in the dynamics of each group with time, we evenly divided the whole time range (7190 time points) into ten periods each covering 9 days and having 719 time points. We then reconstructed the phase space of the average movement counts at each of the ten periods for each group (See Supplementary eFigure 2 for the detailed computation procedure and Supplementary eTable 1 for the delay time τ and embedding dimension τ for each group of mice). Due to space limitations we illustrate here the attractors of three typical periods in Figure 2 and detailed periods are shown in Supplementary eFigure 3. To ease comparisons of these complex shapes, we describe the shape of attractors using specific letters like ‘Z’, ‘T’, ‘V’ and ‘Vs’ (scattered V).

In both the 24AL and 12AL groups, there was no significant change in the shape of

attractors over the 10 periods and the shape consistently looked like the letter 'Z' from the front view. In the 10CR group, the shape of the attractors in the first six periods also looked like a 'Z'; however, it changed to look more like 'T' from period 7 to 10. In the 20CR group, the shape of the attractors from period 1 to 6 looked like 'Z', then changed to 'T' from period 7 to 8, and finally changed to 'V' in the last two periods. In the 30CR group, the shape of attractors looks also looked like 'Z' in the first five periods, however, this changed to 'V' in the last five periods. In the 40CR group, the shape of the attractors looked like 'Z' in period 1 and 'V' from period 2 to 6; after that, it changed to a more scattered 'V', denoted by 'Vs'. See Supplementary eTable 2 for a summary.

So far, we have explored the dynamic characteristics of each group of mice under different CR levels, with different CR levels giving rise to distinct attractors. However, this plotting of attractors only gives a collective and qualitative illustration. We now switch from the qualitative collective analysis to the quantitative individual analysis by investigate three important quantitative characteristics: CD, KE and size of the volume of the phase space for each individual mouse to statistically characterize and distinguish the attractors of the six groups.

2.3. Distinct CR levels exhibit different characteristics (CD, KE and size) of the attractors

In this section, we statistically investigate the characteristics (CD, KE and size) of the attractors of mice as they changed over the ten periods. For this analysis we first computed CD, KE and size of the attractor of each mouse in each period. We then fitted regression models to the parameters over time for each mouse. For KE and CD these regressions were best fit by linear relationships (based on inspection of residuals) but for size the best fit model was a log-linear regression. For each mouse we derived the gradient and intercept values of the fitted regression and the explained variance (r^2). Supplementary eTable 3 summarizes these derived parameters relating to the change over time in CD, KE and size for each mouse in each group. Figure 3 shows how the fitted intercepts and gradients for CD, KE and size differed between

the CR groups. From Figure 3 and Supplementary eTable 3, we obtain the following observations.

For CD there was a significant difference in the fitted intercepts between the groups (ANOVA $F_{5,40} = 3.5$, $p = 0.01$: Figure 3A), post-hoc tukey tests indicated the intercept for 40CR was significantly lower than the intercepts of the 10CR and 30CR groups (both $p < .01$). All other comparisons were not significantly different. The gradient of change in CD over the 10 periods however showed a much greater difference between groups ($F_{5,40} = 32.13$, $p < .0005$: Figure 3B) with the 24AL, 12AL, 10CR and 20CR all having positive gradients that were not significantly different from each other (CD increasing over time on restriction) but the 30CR and 40CR groups having negative gradients (CD decreasing over time) that did not differ between these 2 groups, but were significantly different from the other 4 groups. For KE there was a significant difference in the fitted intercepts between the groups (ANOVA $F_{5,40} = 13.58$, $p < .0005$: Figure 3C). Post-hoc tukey tests indicated the intercepts for 30CR and 20CR groups were significantly higher than the other 4 groups ($p < .05$ for both comparisons). While 12AL and 10CR were significantly lower than the other 4 groups ($p < .05$ for all comparisons). All other comparisons were not significantly different. The gradient of change in KE over the 10 periods however was not different between the different levels of CR ($F_{5,40} = 1.83$, $p = 0.128$: Figure 3D) with all groups showing a slight increase over time. Finally, for size, there was no significant difference in the fitted intercepts between the groups (ANOVA $F_{5,40} = 1.34$, $p = 0.268$: Figure 3E). However, the gradient of change in size over the 10 periods showed a highly significant difference ($F_{5,40} = 61.82$, $p < .0005$: Figure 3F) with the 24AL, 12AL, 10CR and 20CR all having negative gradients that were not significantly different from each other (size decreasing over time on restriction) but the 30CR and 40CR groups having positive gradients (size increasing over time) that did not differ between these 2 groups, but were significantly different from the other 4 groups.

We further explored the state change using the method of coefficient of variation (see Supplementary Methods for the detailed method and Supplementary eFigure 4 for the analysis result), where we can see that there was a large change between 30CR and 40CR.

2.4. Distinct CR levels exhibit different multifractal spectra

Similarly, we divided the whole time series into 10 parts (phases), and computed their multifractal spectra, illustrated in Supplementary eFigure 5, where we can see the time series of each mouse in each group is multifractal as the values of the singular exponent α vary. Moreover, α ranges from 2 to 5 for almost all mice of 24AL, 12AL and 10CR, while α exceeded 5 for almost all mice of 30CR and 40CR and almost 1/2 mice of 20CR.

Furthermore, the singular spectrum $f(\alpha)$ varied considerably from 24AL to 40CR, although the main part of each $f(\alpha)$ looks like an inverted parabola. The MSA results given in Supplementary eFigure 5 generally classify the six groups of mice into two categories, which is consistent with the result given by PSR above (compare Table 1). One category involves 24AL, 12AL, 10CR and 20CR, which have similar singular spectra $f(\alpha)$, i.e., most mice in these groups have a tail for only on the left side. The other category involves 30CR and 40CR, both of which have similar singular spectra, i.e., some mice have a tail on the left part, but each mouse has a tail on the right side. Similarly, different groups of mice show distinct multifractal spectra, which result in distinct phenotype behaviors. Besides, the MSA result also reveals that the behavior of 24AL and 12AL is a bit different, as most mice of 24AL have a tail on the left part in terms of $f(\alpha)$. Moreover, there is a big change from 20CR and 30CR, i.e., the parabola-like curves without a tail (for 20CR) become to have a tail (30CR and 40CR).

2.5. PSR gives accurate predictions of movement counts

Using the PSR technique together with the Volterra adaptive filter, we further achieved an accurate prediction of movement counts over time. We randomly selected a mouse from each group, i.e., 24AL (mouse 3), 12AL (mouse 9), 10CR (mouse 17), 20CR (mouse 25), 30CR

(mouse 34) and 40CR (mouse 45). Each time series of movement counts (in total 7190 time points) was scaled in the range (-1, 1) and divided into two parts: the first 6200 values being the training set, used to reconstruct the model, and the rest treated as the test set. We then applied the above-mentioned technique to the time series, and obtained the plots given in eFigure 6, in which the measured and predicted time series are given and the mean square errors are also given on the right of the plots. From each plot, we can see the error was always lower than 3.6% and hence the PSR technique together with the Volterra adaptive filter gives a precise prediction of future movement counts of the mice. Hence by applying the PSR technique to movement counts, we achieved not only a good characterization of the behavior, but also a precise prediction of their future behaviors.

2.6. Mapping phenotypes to gene expression in the hypothalamus

As illustrated above, different groups of mice under CR produce distinct phenotypes, represented by distinct attractors. For the same individual mice we also have the gene expression profiles in the hypothalamus at the end of the 3 month long restriction period. To explore the relationships between the characteristics of the attractors in individual mice with their gene expression profiles we calculated the correlation coefficients between expression of each gene (normalized counts) and the individual values of the parameters KE and CD in the final period. The genes that correlated significantly (see methods for definition of false discovery cut-offs) with KE and CD are shown in eTable 4. We performed this analysis only for the values of KE and CD for the final time period since this was the point at which the mice were killed and the gene expression measured. Hypothalamic gene expressions of only five genes were correlated with the values of KE, all of which were positively correlated. The genes were *Lbp, Wfikkn2, Gm2518, Msx1* and *Krt18*. In contrast for CD there were 23 significant genes (10 positive and 13 negative). The positively correlated genes were *Fcrls, Tubb2b, Stmn4, Ccnd1, Serpinb1a, Oxct1, Phyhipl, Lmcd1, Ccdc148* and *Ppid*. The significantly negatively

correlated genes were *Pla2g3, Errfi1, Stx3, Cdnk1a, Eif2s3y, Fkbp5, Agrp, Adipor2, Sgk1, Ovgp1, Plin4* and *Hif3a*. Expression levels of these genes were not independent of each other. We performed a stepwise multiple regression (forward inclusion and backward deletion with F to enter or leave = 0.15) to identify the most significant predictors. Although we use the term predictors for this analysis, this should not be taken to imply a direction of causality in the relationship of gene expression to the attractor structure. For CD, four of the 23 genes were retained in the best fit equation. We also performed a best-subsets regression and then selected the best combination of predictors based on minimisation of the Akiake Information criterion (AIC) to avoid overfitting. This also identified the same 4 genes as the best combination of predictors for CD. The four genes were *Tubb2b* (t = 2.79, p = 0.009), *Stmn4* (t = 2.83, p = 0.008), *Agrp* (t = -2.51, p = 0.018) and *Eif2s3y* (t = -2.13, p = 0.042), and together expression of these genes explained 77% of the variation in CD ($F_{4,33}$ = 24.22, p < .005). Relationships between CD and these 4 predictors are shown in Figure 4. For KE the multiple regression analysis identified 2 significant predictors. These were *Wfikkn2* (t = 3.11, p = 0.004) and *Gm2518* (t = 3.9, p = 0.004) and together they explained 56.5% of the variation in KE ($F_{2,33}$ = 20.16, p < .0005). Relationships between these 2 predictors and KE are shown in Figure 4.

3. Discussion

3.1. PSR characterized mouse groups consistent with hierarchical clustering in the time domain

Traditional time domain methods for time series only give a superficial and one-sided understanding of the behavior of a system (compare Figure 1) with partial observations of some properties of a system such as movement counts. To find the underlying mechanism that results in the phenotype change of distinct groups of mice, PSR was used to reconstruct the observed time series of a system and thus reveal the real system's behavior, which clearly shows in each group of mice there is a change of the intrinsic behavior (attractors) with time due to different

CR levels (compare Figure 2 and Figure 3) and this change causes the classification of mice given in Figure 1(b). With PSR, we build a map from the internal attractors to phenotypic behavior (2D plot of time series and classification). It has also been found that attractors refine the classification of mice given by the hierarchical clustering method in the time domain. That is, hierarchical clustering classified the mice into three categories, one consisting of 24AL and 12AL, one of 10CR and 20CR, and one of 30CR and 40CR, which is confirmed by the PSR approach (see Table 1).

3.2. PSR builds a map from internal attractors to phenotypic behavior of mice under distinct CR levels

As indicated by Supplementary eFigure 7, the KE and CD increase, but the size decreases for the 24AL, 12AL, 10CR and 20CR groups, while the KE and size increase but the CD decreases for the 30CR and 40CR groups. This observation first gives an impression that the CR less than 20% does not affect the behavior too much, as the 10CR and 20CR groups have a similar change trend to 12AL. The increasing size of the 30CR and 40CR groups reveals that their movement space increases, which may imply that they move further seeking to find food than other groups of mice. The decreasing CD of the 30CR and 40CR groups indicates a less geometric complexity than other groups, which means less forms of behavior these two groups take. In this case, these two groups of mice may have to save energy and only concentrate on food searching due to insufficient food intake. Besides, the increasing KE of the 30CR and 40CR groups implies that these mice become more chaotic, which means the bahavior of these mice becomes more irregular and unpredictable.

In summary, from Figure 3 and Supplementary eFigure 7 and eTable 3, we can see that the mice under distinct CR undergo significant changes of their underlying mechanism reflected by their attractors' characteristics, CD, KE and size. It is the change of the underlying mechanism that results in the change of phenotypes of mice under distinct CR and thus the

classification of mice given in Figure 1(b). With PSR, we build a map from the internal attractors to phenotype behavior (2D plot of time series and classification). Multifractal spectrum analysis further validates this mapping.

3.3. Links of the attractor parameters CD and KE to the gene expression profiles in the hypothalamus

There were only five genes significantly correlated with the values of KE, all of which were positively correlated. The most significantly correlated gene was lipopolysaccharide binding protein (*Lbp*) involved in the immune response to bacterial infection. Its role in this context is unclear. The second most correlated gene expression was with the wap follistatin/kazai immunoglobulin kunitz and netrin domain containing 2 (*Wfikkn2*), a gene that contains multiple binding domains and may be a regulator of proteases. It is known to bind to tumor growth factor beta and bone morphogenic proteins 2 and 4, although apparently does not affect their signaling activity [40]. The third highest correlation was with expression of *Gm2518* which may be a pseudogene. Mouse homeobox 1 (*Msx1*) was the fourth highest correlated gene expression to KE. This is a transcriptional regulator during development and is also expressed in the pituitary. Its potential function in this context is unclear. Finally, the firth most correlated gene was keratin18 *(Krt18*). This gene is expressed in a wide range of tissues although not previously suggested to be a feature of hypothalamic gene expression. Again the function in this context is uncertain. The multiple regression analysis suggested that the two most important genes influencing KE were *Wfikkn2* and *Gm2518.*

There were 10 genes positively related to the level of CD. The most highly correlated gene to CD was *Fcrls*. This is a receptor expressed on microglia but it has no known ligand and no known function. The gene contains immunoglobulin domains and hence has been suggested to be linked to brain inflammation. Discovery of the function of this gene and its ligand should be a key goal because it may play a significant part in structuring the behavioral response to CR

and potentially therefore also the life and healthspan benefits. In fact, 2 other genes in the top 10 positively correlated genes are also associated with inflammation: *Serpinb1* which is a serine protease inhibitor, and strathmin 4 (*Strm4*) which is involved in microtubule destabilization. This gene is known to be regulated by leptin which shows large changes under CR (12). A microtubule link is also evident for the second most positively correlated gene to CD and that was Tubulin (*Tubb2b*) which binds GTP as a major component of microtubules. Mutation of this gene leads to cognitive impairment. The 4th highest correlation to CD is for expression levels of cyclin D1 (*Ccnd1*). This is a key gene involved in regulation of the cell cycle. Why it should be linked to this bahavioral parameter is unclear at present. The 6th most positively correlated gene was *Oxct1* (succinyl coA ketoacid coA transferase), involved in the breakdown of ketones. This is potentially significant because animals under the highest levels of restriction may be involved in cyclic utilization of ketones during the daily cycle. Such ketone use may be a driver of the attractor structure. The 7th most significant positive gene was *Phyhypl* (phytanoyl coA 2 hydroxylase interacting protein) involved in brain specific BAI-1 binding and regulation of endogenous siRNAs. This may then be a key regulator of other genes that control the behavior. The 10th highest positively correlated gene was *Ppid* (Cyclophilin d1) which has been linked to motor dis-cordination and autism spectrum disorder, hence also potentially involved some presently unknown manner in physical activity regulation. The two other genes significantly positively associated to CD were *Ccdc148* and *Lmcd1*. The former has unknown functions in the brain, and the latter is associated with restricting GATA function.

Among the genes that were negatively correlated to CD the most negatively correlated was *Hif3a* (hypoxia inducible factor 3a). This gene is typically upregulated in conditions of hypoxia where it mediates the adaptive response. Why it should be strongly related to the attractor underpinning the bahavioral response to CR is unclear. The second and third genes *Plin4* (perilipin4) and *Arrdc2* (arrestin domain containing 2) also have well established functions. The

former encodes a protein that coats lipid droplets, while the latter regulates g-protein coupled signaling and cargo protein trafficking in endo-lysosomal system. Again why these might link to bahavioral responses to CR is unclear. The 5th negatively associated gene was *Sgk1* (serum glucocorticoid regulated kinase 1). Since this responds to glucocorticoids, which are modulated by CR, this gene may form a link between circulating levels of glucocorticoids and the bahavioral response. This is also true for 2 other genes that are significantly negatively related to CD. These are the adiponectin receptor (*Adipor2)* in 6th position and Agouti-regulated peptide *Agrp* in 7th position, which is regulated by both leptin and insulin. These three genes may provide an important link between peripheral signals reflecting the CR state and the bahavioral response. *Sgk1* is also involved in the cellular stress response and this is also true for *Errfi1* (erbb receptor feedback inhibitor 1) in 12th place. The 8th most negatively correlated gene was *Fkbp5* (FK506 binding protein 5) which is an immunophilin protein. *Stx3* (Syntaxin3) was the 11th most negatively correlated gene to CD and is related to protein trafficking. Finally, *Cdkn1a* in 10th position is a cyclin dependent kinase regulating the cell cycle at G1, clearly linked to Cyclin D1, which appears in the positive related list. The function of the ninth most negatively correlated gene (*Eif2s3y*) in the brain is unknown.

These genes therefore include 4 genes linked to peripheral hormones that may mediate the CR state to the bahavioral response (*Sgk1, Agrp, Adipor2* and *Stmn4*), two genes linked to the cell cycle regulation (*Ccnd1* and *Cdkn1a*), two genes linked to microtubules (*Tubb2d* and *Stmn4*) and three genes potentially linked to inflammation (*Fcrls1, Serpinb1a* and *Stmn4*). It is important to recognize that the directional in causality in their associations to the attractors for all these genes is unknown. Hence the genes may cause the behavior changes, or the changes in the gene expression may be a consequences of the attractor structure. At present we cannot separate these possibilities. Some of the other genes have well known functions but why they should provide any link to the bahavior is unclear (eg *Hif3a* and *Plin4*) while others do not have

a known function in the brain at present. It is important to recognize that these associations are only correlations and no causality can be inferred. The multiple regression analysis indicated that perhaps the most significant of these genes were *Tubb2b, Stmn4, Agrp* and *Eif2y3*. Now we have identified these potentially important genes it will be interesting to experimentally manipulate these genes in the brain using genetic techniques to evaluate their causative role in the mediation of the attractor structure.

4. Methods

4.1. Overall design and rationale

All the experiments were performed at the University of Aberdeen, UK under Home office project licence (PPL 60/4366 held by JRS), following ethical approval of the protocols by the local ethical review committee. Male 6 week old C57/BL6 mice (Charles River, Ormiston, UK) were acclimated for 6 weeks and then transmitters were implanted at 12 weeks of age. The physical activities registered as movement counts, were measured using the VitalView ™ telemetry and data acquisition system (MiniMitter, OR, USA). The transmitters, implanted intraperitoneally, are unrestrictive and allow completely free movement of the mice. The data are transmitted via an ER-4000 receiving platform and VitalView TM software was used to acquire data (MiniMitter, OR, USA).

The 48 mice were randomly divided into six experimental groups (24AL, 12AL, 10CR, 20CR, 30CR and 40CR), with eight mice in each group. Mice were kept at room temperature (21-23^{o}C) on a 12:12 photoperiod and fed a commercial diet providing 20% calories as protein and 10% calories as fat (Research diets: D12450B) 24AL means the mice were fed completely *ad libitum* without restriction for 24 hours per day; 12AL refers to *ad libitum* feeding for 12h per day during darkness. 10CR, 20CR, 30CR and 40CR refer to caloric restriction by 10%, 20%, 30% and 40%, respectively, relative to the baseline intake of the same individual mouse. All mice were exposed to the same feeding regime (12AL) during the baseline period of two

weeks and were then exposed to CR for a period of 12 weeks starting at age 20 weeks. For further details of the experimental rationale, see [11]. We do not have information on the time it took the different groups to ingest their food. It is possible that those mice who were on greater levels of restriction ate their food more quickly, and hence the level of restriction is confounded with the period of each day that the mice were fasting. There is no data at present to support or refute this possibility.

We removed the data for two mice where the transmitter malfunctioned before the end of the experiment leaving 46 mice in total. We recorded the movement counts at 7190 time points after the baseline period. Each time point reflected the accumulated counts over 15 minutes. The original mice were randomly allocated and therefore had random numbers but to facilitate the presentation in the following sections, we renumbered all the included mice as follows. That is, we number the mice from 1 to 8 for 24AL, 9 to 16 for 12AL, 17 to 24 for 10CR, 25 to 32 for 20CR, 33 to 38 for 30CR, and 39 to 46 for 40CR.

4.2. Phase space reconstruction (PSR)

PSR is one of main approaches to analyse nonlinear time series and can generate the attractor of a system, a subset of the system's phase space, which may be considered as a geometric object to which all the asymptotic states of the system's trajectories belong [28-31]. A detailed description of PSR is given in Supplementary Methods.

4.3. Correlation dimensions and Kolmogorov entropy of attractors

Correlation Dimension (CD) is an important measure of an attractor in the phase space, and it characterises the geometric complexity of the attractor [32-34]. A bigger CD means a more complex geometry of an attractor. Kolmogorov entropy (KE) quantifies "how complex or chaotic" a signal is [35,36]. A detailed description of CD and KE is given in Supplementary Methods.

4.4. Multifractal spectrum analysis

Multifractal describes the characters of different scales when a fractal geometric body grows, and multifractal spectrum analysis (MSA) is an important method for complex dynamic systems with multi-fractal features [37-38]. A detailed description of MSA is given in Supplementary Methods.

4.5. Time series prediction with Volterra adaptive filter

We used the Volterra adaptive filter [39] to predict the movement counts of mice, which is described in Supplementary Methods.

4.6. Correlation of KE and CD to hypothalamic gene expression

The detailed description of the establishment of the key genes that are associated with the attractor characteristics [40] is given in Supplementary Methods.

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FL, LC and JRS conceived the project. DS performed the phase space reconstruction and associated analysis. JRS and DD performed gene related analysis. SM performed the mouse experiment. DS, FL, JRS and LC wrote the main part of the manuscript. All authors contributed to the writing of this manuscript.

Conflict of Interest

The authors confirm that no conflicts of interest exist.

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Table 1. A map between the characteristi cs of attractors (and multifractal spectra) and the behavioural phenotypes.

Group	**Attractor characteristics**				**Multifractal spectra(curves)**	**State**
	Shape	KE	CD	Size		
24AL	**Z→Z→Z**	KE↑	CD↑	size↓, small variance	Most mice have a tail on the left part	Normal attractor
12AL	**Z→Z→Z**	KE↑	CD↑	size↓, small variance	Some mice have a tail on the left part	Normal attractor
10CR	**Z→Z→T**	KE↑	CD↑	size↓, big variance	Some mice have a tail on the left part	Almost normal attractor
20CR	**Z→T→V**	KE↑	CD↑	size↓, big variance	Some mice have a tail on the left part	Almost normal attractor
30CR	**Z→Z→V**	KE→	CD↓	size↑, bigger variance	Each mouse has a tail on the right part	New attractor
40CR	**Z→V→Vs**	KE↑	CD↓	size↑, bigger variance	Each mouse has a tail on the right part	New attractor

Figure 1. Time series and hierarchical clustering analysis of movement counts of mice. (a) Average movement counts of each of six groups of mice with time. These average time series show differences of these groups, especially between the first four and the last two groups. (b) Hierarchical clustering analysis of 46 mice, which classifies the mice into three categories. Specifically, the blue category mainly consists of the mice from 24AL (8/20) and 12 AL (8/20), the green category mainly from 10CR (5/10) and 20CR (4/10), and the red category mainly from 30CR (5/16) and 40CR (8/16). This also reveals that mice under different levels of CR exhibit distinct behaviour time series. In our experiment, we divide the mice into six groups, i.e, 24AL, 12AL, 10CR, 20CR, 30CR and 40CR. 24AL means the mice were fed completely ad libitum without restriction for 24 hours per day; 12AL refers to ad libitum feeding for 12h per day during darkness. 10CR, 20CR, 30CR and 40CR refer to caloric restriction by 10%, 20%, 30% and 40%, respectively, relative to the baseline intake of the same individual mouse.

Figure 2. The evolution of the dynamics (attractors) of each group of mice with time, illustrated with typical three periods (1, 7, 9 for the first five groups and 1, 5, 9 for the 40CR group) from left to right. We divide the total 7190 time points into 10 periods, each occupying 719 time points, and here illustrate the attractors of typical 3 periods. From each row, representing a group, we can see the attractors evolve smoothly for 24AL and 12AL, and even for 10CR and 20CR, but significantly differently for the rest groups. To ease comparisons of these complex shapes, we describe the shape of attractors using specific letters like ‘Z’, ‘T’, ‘V’ and ‘Vs’ (scattered V).

Figure 3. Evolution of the characteristics of attractors over the ten time periods. For each individual we fitted a linear regression of the attractor values (CD, KE and $\log_e$ Size) against

time over the ten time periods. The derived intercepts and gradients of these regressions are shown here in relation to the level of restriction. Each point is a different individual. A: intercept CD. B: gradient of change in CD, C: intercept KE, D: gradient of change in KE. E: intercept $\log_e$ size and F: gradient of change in $\log_e$ size. For CD there was a significant difference in the fitted intercepts between the groups (ANOVA $F_{5,40} = 3.5$, $p = 0.01$: Figure 3A), post-hoc tukey tests suggested the intercept for 40CR was significantly lower than the intercepts of the 10CR and 30CR groups. The gradient of change in CD over the 10 periods however showed a much greater difference between the groups ($F_{5,40} = 32.13$, $p < .0005$: Figure 3B) of 24AL, 12AL, 10CR and 20CR and the groups of 30CR and 40CR. For KE there was a significant difference in the fitted intercepts between the groups (ANOVA $F_{5,40} = 13.58$, $p < .0005$: Figure 3C). The gradient of change in KE over the 10 periods however was not different between the different levels of CR ($F_{5,40} = 1.83$, $p = 0.128$: Figure 3D) with all groups showing a slight increase over time. For size, there was no significant difference in the fitted intercepts between the groups (ANOVA $F_{5,40} = 1.34$, $p = 0.268$: Figure 3E). However, the gradient of change in size over the 10 periods showed a highly significant difference ($F_{5,40} = 61.82$, $p < .0005$: Figure 3F) between the groups of 24AL, 12AL, 10CR and 20CR and the groups of 30CR and 40CR.

Figure 4. Bivariate plots of the relationships between the expression levels of 4 genes expressed in the hypothalamus (Tubb2b, Strm4, Agrp and Eif2s3y) and the correlation dimension (CD) of the attractor and 2 genes (Wfikkn2 and Gm2518) and the Kolmogorov entropy (KE) of the attractor.