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[http://dx.doi.org/10.1016/j.bbr.2013.09.004](http://dx.doi.org/10.1016/j.bbr.2013.09.004)
We thank the reviewers for their poignant and constructive comments on our manuscript and address each of the comments and concerns below.

**Reviewer 1:**

**Introduction:**

‘much’ is deleted from the first line of the introduction.

**Methods:**
The treadmill used was a custom built 8 lane exercise treadmill with 5% slope supplied by another investigator in Medical Sciences here at RMIT University, Professor John Hawley. Unlike some treadmills used in experimental procedures there was no aversive stimulus i.e. a shock apparatus if the animal slowed down or stopped running. Instead the rat slid off the back of the treadmill into a soft catchment area. We could then place the rat back on the treadmill. After piloting a number of speeds and timings we found that our rats would run for a maximum of 15 minutes before they began to have episodes of refusing to run. As such we exercised twice day as we were looking for a 30min/day training regime which was recommended by our animal ethics committee and Professor Hawley, an exercise physiologist, with numerous publications using this apparatus:


We have now included one of these references in the manuscript.

**Results:**
Figures 1 and 2 (now 3) have been amended (bolded font, thicker lines). An example of PV staining has been added to figure 3.

**Discussion:**

1. As suggested by the reviewer we have elaborated on our elevated plus maze results in the discussion, furthering this to the anxiolytic effect of exercise, and indeed environmental enrichment (paragraph 4 of discussion).
2. As requested a figure of the elevated plus maze data has been included (now figure 2).
3. As recommended by the reviewer we have included some discussion on the possible association of PV staining and social behaviour.
4. Unfortunately we do not have any further brain sections from this cohort of animals to do further PV staining. However we believe that our hippocampal analysis demonstrates consistent findings with other researchers investigating this class of neurons and voluntary or treadmill exercise (Hwang, 2011; Arida, 2004; Gomes da Silva, 2010), thus replicating the effect of the intervention.
5. A more definite conclusion has been added.

**Reviewer 2:**

**Major comments:**

1. The reviewer was correct in that there was an error with figure 1. This has now been amended. All data has also been rechecked and is correct.
2. Control rats were handled for approximately five minutes each. This has been added to the methods. Indeed sitting the control rats on the stationary treadmill for the same amount of
time as the exercising animals would be a very good idea. We thank the reviewer for this suggestion and will comply with this in the next study. Rats were run on the treadmill with their cage mates and thus the fellow runners were not novel. This detail has now been added to the methods.

3. We have chosen a low intensity exercise protocol for a number of reasons:
   a. We were not interested in any kind of development in increased muscle physiology;
   b. We were looking to in part model the exercise observed in an intervention like environmental enrichment;
   c. As discussed above in the methods section of reviewer one after piloting a number of speeds and timings we found that our rats would run for a maximum of 15 minutes before they began to have episodes of refusing to run. As such we exercised twice day as we were looking for a 30min/day training regime;
   d. This regime was recommended by our animal ethics committee.

Minor comments:
1. Abbreviations and units of measure have been changed.
   a. Metres have been changed to m.
   b. Minutes have been changed to min.
   c. Hours have been changed to h.
   d. CA2&3 have been changed to CA2/3
   e. + have been changed to plus

2. Time spent in the arms of the elevated plus maze amended in text to seconds

3. References have been amended.
EFFECT OF LOW-INTENSITY TREADMILL EXERCISE ON BEHAVIOURAL MEASURES AND HIPPOCAMPAL PARVALBUMIN IMMUNOREACTIVITY IN THE RAT

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Abstract

Exercise has been demonstrated to have positive effects on both the body and brain. The present study aimed to determine the behavioural and morphological consequence of low-intensity running. Rats were exercised on a treadmill for a total of 30 days, 30 min/day. Social interaction, locomotor activity and behaviour on an elevated plus maze were assessed post-treatment. Exercised animals demonstrated more passive interaction and less time not interacting than control animals that were not exercised. Conversely, locomotor and anxiety measures showed no effect of exercise. Analysis of brains demonstrated an increase in expression of parvalbumin immunoreactive neurons in the hippocampus localised to the CA1 and CA2/3 regions. These results demonstrate that low-intensity exercise leads to changes in social behaviour as well as neuroplastic morphological changes within the hippocampus.

Keywords

exercise, parvalbumin, hippocampus, anxiety, social interaction, locomotion
While it is well recognised that exercise has numerous beneficial effects on the body, recent research suggests that these positive effects also extend to the brain. Clinical studies indicate improvements in cognition in cognitively-normal [1] and impaired adults [2], with increases in brain volume, cerebral blood flow and functional brain plasticity observed. Much of these findings have been replicated in laboratory animals, demonstrating an important role of exercise in inducing positive brain changes in the rodent [3].

γ-aminobutyric acid (GABA)-ergic inhibitory neurons containing the calcium binding protein, parvalbumin, are suggested to be involved in various higher brain functions including emotion, anxiety, and learning and memory [4]. These neurons synapse on the cell body or axon initial segment of glutamatergic neurons and can regulate pyramidal cell output. Indeed a strong correlation has been demonstrated between the presence of parvalbumin and the fast firing properties of hippocampal neurons [5]. Parvalbumin containing GABAergic interneurons are effective markers of hippocampal cells [6] and are demonstrated to increase in number in the hilus of dentate gyrus in rats submitted to acute physical exercise [7].

The effect of exercise on so-called mood measures is less well understood. We know that exercise increases β-endorphin levels [8] and neurotransmitter release, including serotonin and dopamine [9, 10]. Behaviourally, regular moderate physical activity induces improvements in quality of life measures in normal adults and positively influences depressive symptoms in patients [11]. Positive social functioning is an important component of quality of life measures, and is often impaired in psychiatric disorders [12]. To date the social interaction test [13] is widely used by researchers to investigate animal social behaviour in response to novel interventions and treatments. The positive effects of exercise in this task are yet to be identified.

In the present study we evaluated the effect of exercise on social interaction in the rat, and assessed its effect on anxiety and locomotor measures. In addition, we determined if our exercise regime successfully induced morphological changes in the hippocampus, as per previous published reports [7, 14].
Male Long-Evans rats (n=19, Monash University, Australia), weighing 221-271g were used for experimental behavioural testing and subsequent brain analysis. A further 10 Long-Evans rats (Monash University, Australia), weighing 239–293g at the beginning of the study, were used as target animals for the social interaction task. Rats were housed 4-5 to a box under a 12 h light/ 12 h dark (lights on 6am) photoperiod cycle with food and water *ad libitum* in the home cage (57 long x 38cm wide x 20cm high). Room temperature (21 ±1°C) and humidity (30–70%) were kept constant. The experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 and with approval from the RMIT University Animal Ethics committee.

Exercise rats (EX, n=10) were habituated to a treadmill for a total of 30min on 3 subsequent days. Exercise rats ran for a total of 30days at 5m/min 5% incline for the first 5days and then progressively trained to run 10m/min 5% incline bi-daily (at 0900h and 1530h) for a cumulative total of 30min for 5weeks (protocol amended from [15]). The treadmill was a purpose built 8 lane exercise treadmill with dividing walls suspended over the tread surface and was cleaned with 70% alcohol after each rat’s exercise session. EX rats were run in teams with their cage mates. Control rats (CON, n=9) were handled daily for approximately 5 min each. At the end of this training period behavioural analysis commenced. All testing was performed during daylight hours (between 9am and 3pm) under 300lux lighting in a randomised cohort order in the below test order with 24h between tests.

In the social interaction test an EX or CON rat was placed in the test arena (black wooden box, 60cm long x 60cm wide x 50cm high, rats placed nose to opposing corners) with a previously unknown ‘target’ rat for 10min to observe social interaction. All sessions were videotaped for analysis of active social interaction (sniffing, grooming, following, or crawling over/under within 5cm of the target rat), passive social interaction (defined as the experimental rat being within 2cm of the target rat but not actively interacting) or no interaction, was carried out for the 10min interaction period [13, 16]. Experimenters were blind to treatment condition.

The elevated plus maze, elevated 70cm above the ground, consisted of two open arms (70x10cm) and two enclosed arms (70x10cm) with a 25cm high surrounding clear Perspex wall, with the arms
extending from a central platform (13x13cm). Rats were placed individually on the maze in the centre platform, nose facing the closed arm. Behaviour was measured for 5min and videotaped for subsequent analysis of arm entries and time in arms. Experimenters were blind to treatment condition.

Locomotor activity was performed using a Med Associates (USA) open field test chamber (44.5cm x 44.5cm x 30.5cm) as previously described [17]. Rats were placed individually in the test chamber for 10mins with analysis of total distance travelled and average velocity performed by Med Associates (USA) activity monitor software, version 4.

Rats received a lethal dose of sodium pentobarbital (1ml/kg body weight). Brains were removed with the left hemisphere fixed in 4% paraformaldehyde in PBS and the right hemisphere frozen in isopentane (Sigma-Aldrich) cooled to -35°C by dry ice. Serial coronal sections (30µm) from the left hippocampus (bregma –3.3mm) were processed for parvalbumin immunohistochemistry as previously described [16]. In brief sections were incubated in 0.6% hydrogen peroxide solution followed by 5% normal horse serum with 0.1% Triton X-100. Following a 36hour incubation with parvalbumin antibody (Swant, Switzerland; 1:10,000) and 2hour incubation in biotinylated anti-mouse IgG, sections were processed by the avidin-biotin method using a Vectastain ABC kit (Vector Laboratories, UK) and visualised using 3’, 3’–diaminobenzidine (DAB) intensified with nickel chloride. Sections were mounted and allowed to dry overnight before being dehydrated and coverslipped.

For image analysis sections from each rat were captured at 40x magnification using a Nikon Eclipse 90i microscope interfaced with NIS-Elements Advanced Research 3.21.000 (Build 689) via a Nikon D-Eclipse C1 camera. Manual counting of parvalbumin-immunoreactive neurons using Image J was carried out for dentate gyru (DG), CA1 and CA2/CA3 subregions, with the entire extent of the target region within the selected coronal sections assessed. Counts were taken from at least four alternate sections from each hemisphere, and these counts then averaged to produce a mean.

All data are presented as mean±standard error of the mean (SEM). Social interaction, elevated plus maze data and parvalbumin levels are analysed by multivariate ANOVA (SPSS, version 19, IBM,
USA) and where appropriate with simple effects. Locomotor activity was assessed by t-test (SPSS, version 19, IBM, USA).

Low intensity treadmill exercise had a significant effect on sociability behaviour. While there was no overall observed effect of group ($F_{(1,17)}=2.6$, $p=0.13$), a significant difference in the type of behaviour ($F_{(1,17)}=60.9$, $p<0.0001$) and group x behaviour interaction ($F_{(1,17)}=7.4$, $p<0.05$) was observed. Posthoc analysis showed that EX animals did not differ in their active behaviour scores (F<1), but were more likely to passively interact with the target rat, that is to be in close proximity to it ($F_{(1,51)}=4.8$, $p<0.05$) without moving away and avoiding contact with it ($F_{(1,17)}=13.5$, $p=0.005$) than the CON animals who were not exercised (Figure 1).

Exercise did not affect anxiety levels with EX and CON animals performing similarly on the elevated plus maze. Considering arm entries (open: EX 4.2±0.4, CON 3.2±0.4; closed: 6.8±0.4, CON 6.3±0.5) there was no group ($F_{(1,17)}=2.2$, $p=1.6$), nor group x arm interaction (F<1). Both groups of rats had significantly more entries into the closed arms than the open ($F_{(1,17)}=45.8$, $p<0.001$) and this was not different between the EX and CON rats (F<1). In addition, for time (sec) spent in the arms (open: EX 82.4±11.3, CON 71.0±9.8; closed: 217.6±11.3, CON 229.0±9.8), there was no group (F<1), nor group x arm interaction (F<1). Both groups of rats spent significantly more time in the closed arms than the open ($F_{(1,17)}=90.2$, $p<0.001$) and again this was not different between the EX and CON rats (F<1) (Figure 2).

All rats exhibited equivalent basal locomotor function irrespective of being exposed to low intensity exercise (distance travelled: EX 1527±132, CON 1598±127; $p=0.71$; average speed: EX 47.6±2.2, CON 49.9±8.6; $p=0.52$).

Exercise significantly increased the number of parvalbumin-immunoreactive neurons in the hippocampus. Significant changes were observed between EX and CON ($F_{(1,16)}=14.0$, $p<0.01$); hippocampal region ($F_{(1,16)}=197.0$, $p<0.001$), and their interaction ($F_{(1,16)}=9.0$, $p<0.01$). Posthoc analysis showed that EX animals had significantly raised parvalbumin immunoreactivity in CA1 ($F_{(1,48)}=34.7$, $p<0.001$) and CA2/3 region ($F_{(1,48)}=5.5$, $p<0.05$), but not DG (F<1) (Figure 3).
This study demonstrates for the first time that low-intensity treadmill running has significant effects on social behaviour in the rat. This effect is observed with no concurrent changes in general anxiety or locomotor measures, but with an observed increase in parvalbumin-immunoreactive hippocampal neurons.

Social interaction measures social and explorative behaviours between unfamiliar pairs of rats and has been repeatedly validated as a measure of anxiety-related behaviour, assessing both anxiolytic and anxiogenic effects [13]. Treadmill exercise produced a reduction in the social anxiety observed when pairing an exercised animal with a previously unknown ‘target’ animal. We observed less social withdrawal in the EX animals in response to the ‘target’ rat compared to unexercised controls, and an increase in ‘passive’ interaction where there is a higher level of non-active contact with the unknown ‘target’ rat. This observed increase in social interaction, observed without an associated increase in locomotor activity or difference in movement on the elevated plus maze, is indicative of an anxiolytic effect of exercise on social behaviour [13].

While there is much anecdotal qualitative evidence observed in humans of the benefits of exercise in decreasing anxiety and other psychiatric symptoms [12], this is the first observation of improved sociality after treadmill exercise in normal rats. Indeed the observations of any exercise on social interaction in rodents is limited and varied: two week voluntary exercise in mice also produced an increase in social contact [18], conversely in Spontaneously Hypertensive Rats a similar exercise regime reduced the number of social interactions, at least in females [19].

When using the elevated plus-maze test we demonstrated no differences in behaviour between our EX and CON rats. This is consistent with other reported studies using low intensity physical treadmill training, as we have used here, in so-called ‘normal’ adult rats [20, 21]. However it is clear that beneficial effects of exercise are observed in animal models of impairment in terms of reducing anxiety. Exercise has been reported to decrease anxiety in aged rats [22]; rodent models of neurological disease such as Parkinson’s disease [23] and attention hyperactivity deficit disorder [24]; and stressed rats [25]. Moreover, it must be recognised that environmental enrichment, which
has a large voluntary exercise component, consistently produces an anxiolytic effect in rodents [26, 27].  

Treadmill training in rodents offers a consistent exercise protocol between subjects. Our results in both the social interaction and elevated plus-maze negate the suggestion that forced treadmill running may issue a long-term physiological anxiety or stress response, which is validated by studies showing no difference in plasma corticosterone or adrenocorticotropic hormone levels in rats between forced and voluntary running [28]. Moreover the beneficial effects of endurance training on the brain suggests its importance in neuroprotection, including antioxidative properties [29] and decreases in neuronal apoptosis [30].

We demonstrate an increase in parvalbumin immunoreactive neurons in the CA1 and CA2/3 regions of the hippocampus after training on the treadmill. This is consistent with the findings of other researchers investigating this class of neurons and voluntary or treadmill exercise [7, 14, 31]. Parvalbumin inhibitory interneurons are essential for information processing and play a crucial role in controlling excitatory transmission in the hippocampal neurons [32]. Indeed, exercise is well recognised to enhance hippocampal neurogenesis [3].

A possible association between the increased pattern of PV staining in our exercised group and the anxiolytic results obtained from the social behaviour test are difficult to interpret at this stage. We have previously demonstrated impaired social behaviour, exhibited by increased following of the ‘target’ rat, after subchronic phencyclidine administration that is also accompanied by a decrease in PV-labelled hippocampal neurons in rats [16]. In a recent study prenatal ethanol exposure with neural stem cell treatment reversed a deficit in both PV-labelled GABAergic interneurons and social interaction behaviour [33], suggesting a role for PV-containing GABAergic interneurons in the behavioural abnormalities of social dysfunction.

The beneficial effects of exercise on brain health are widely recognized. Clinical studies have highlighted that exercise enhances cognitive functions, reduces the risks of age-related cognitive impairment [1], and exerts antidepressant effects in mildly to moderately depressed [11]. Social withdrawal and loss of parvalbumin immunoreactive neurons are one of the primary pathologies
evident in schizophrenia [34] and also in bipolar disorder [35] suggesting that physical exercise may have important positive physiological as well as psychological effects in psychiatric disorders [12].

In conclusion, the present study has shown that low intensity treadmill exercise has anxiolytic effects on social behaviour, while concurrently increasing neuron numbers within the hippocampus; demonstrating further the positive effects of exercise on the brain.
Acknowledgements

Jason Nguyen holds an Australian Postgraduates Award (APA) Postgraduate Scholarship. We thank Professor Hawley for the use of the rat treadmill.
Figure Legends

Figure 1
Social interaction after low impact treadmill exercise of exercised (EX) and control (CON) rats. Data are expressed as mean interaction scores±SEM, n = 9-10/group. *p <0.05; ***p=0.001.

Figure 2
Elevated plus maze data after low impact treadmill exercise of exercised (EX) and control (CON) rats. A: Number of arm entries in open and closed arms. B: Time spent in open and closed arms (sec). Data are expressed as mean±SEM, n = 9-10/group.

Figure 3
A: Number of Parvalbumin immunoreactive neurons in the CA1, CA2/3 and dentate gyrus (DG) regions of the hippocampus of exercised (EX) and control (CON) rats. Data are expressed as mean number of immunoreactive neurons±SEM, n = 8-10/group. *p<0.05; ***p<0.001. B: Parvalbumin immunoreactivity in the rat hippocampus of EX rats. C: Parvalbumin immunoreactivity in the rat hippocampus of CON rats. Brightfield photomicrograph of coronal section showing the distribution of parvalbumin immunoreactivity throughout the hippocampus. 4× magnification. Arrows designate parvalbumin-labelled neurons.
References


[34] Zhang ZJ, Reynolds GP. A selective decrease in the relative density of parvalbumin-immunoreactive neurons in the hippocampus in schizophrenia. Schizophrenia research. 2002;55:1-10.

Figure 2

Bar charts showing:

A. Arm entries
- EX
- CON

- Open
- Closed

B. Time in arm (sec)
- EX
- CON

- Open
- Closed