EFFECT OF SPONTANEOUS ACTIVITY AT THE OLD AGE ON ADULT HIPPOCAMPAL NEUROGENESIS IN LRT AND HRT RATS

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TIIVISTELMÄ

Mira Romo (2017). Myöhemmällä iällä aloitetun spontaaniaktiivisuuden vaikutukset hippokampuksen neurogeneesiin LRT ja HRT rotilla. Liikuntatieteellinen tiedekunta, Jyväskylän yliopisto, liikuntafysiologian pro gradu –tutkielma, 61 s.

Fyysinen aktiivisuus on hyväksi aivoterveydelle ja lisää aikuisiällä tapahtuvaa aivoturson eli hippokampuksen neurogeneesiä. Fyysisestä harjoittelusta saatavat vasteet riippuvat kuitenkin osittain geneettisistä tekijöistä ja siksi fyysisen harjoittelun vaikutukset ovat yksilöllisiä. Tämän tutkielman tarkoituksena on selvittää valikoivasti jalostettujen rottalinjojen spontaania fyysistä aktiivisuutta ja liikunnan vaikutuksia aivoterveydelle, kun fyysinen aktiivisuus on aloitettu myöhäisellä iällä pitkän inaktiivisuuden jälkeen. Tutkimuksessa käytettyjen rottien ikä vastaa noin 45 vuoden ihmisikää. Tutkimuksen rotat eroavat harjoitteluun reagoivuuden osalta ja omaavat joko korkean tai matalan harjoitteluvasteen. Rotat (N=79) jaettiin kahteen tutkimusryhmään, joista toiseen ryhmään kuuluneilla oli käytössään juoksupyörä. Rottien spontaaniaktiivisuutta tarkasteltiin sukupuolen, jalostetun rottalinjan ja tutkimusryhmän mukaan. Spontaania aktiivisuutta mitattiin voimalevyjen avulla. Tulosten perusteella rotat ovat aktiivisempia yön ja hämärän aikaan ja naarasrotat ovat huomattavasti urosrottia aktiivisempia. Naaraiden korkeampi aktiivisuus näkyy läpi päivän. Spontaaniaktiivisuudessa ei ollut tilastollisesti merkitseviä eroja rottalinjojen välillä, vaikka korkean harjoitteluvasteen rotat näyttäytyivät aktiivisempina. Vastoin hypoteesia, spontaaniaktiivisuus ei korreloinut hippokampuksen neurogeneesin kanssa eikä neurogeneesi ollut suurempaa HRT rotilla.

Asiasanat: spontaani aktiivisuus; LRT, low responder rat; HRT, high responder rat; peritty ero fyysiseen harjoitteluun, neurogeneesi, hippokampus

ABSTRACT

Mira Romo (2017). Effect of spontaneous activity at the old age on adult hippocampal neurogenesis in LRT and HRT rats. Faculty of Health and Sport Sciences, University of Jyväskylä, Master's thesis in exercise physiology, 61 pp

Physical activity is beneficial for brain health and it increases adult hippocampal neurogenesis. The response to physical activity depends partly on genetic factors and due to that, is individual. The aim of this thesis was to examine the spontaneous activity of selectively bred rat lines and the effects of exercising on brain health when activity is started after a long period of being inactive. The age of the rats used in this study correspond with the human age of approximately 45 years. The rats differ with their responsiveness to exercise and have either high or low responsiveness. Further, the correlation between spontaneous activity and adult hippocampal neurogenesis was examined. The rats (N=79) were divided to two study groups, rats in the second group had running wheels in their cages. Rats' spontaneous activity was analyzed according to sex, line and study group. Spontaneous activity was measured by using ground reaction force plates. According to the results, the rats are more active during night and dusk and the female rats are remarkably more active than male rats. The females are more active throughout the day. There was no statistical difference in the activity between lines, even though rats with high responsiveness appeared to be more active. Against hypothesis, the spontaneous activity did not correlate with hippocampal neurogenesis and neurogenesis was not more present in HRT rats.

Key words: spontaneous activity, LRT, low responder rat, HRT, high responder rat, inherent responsiveness to aerobic training, adult hippocampal neurogenesis

ABBREVIATIONS

AHN	adult hippocampal neurogenesis
BDNF	brain-derived neurotrophic factor
BMAL1	Brain and Muscle ARNT Like 1 protein
CLOCK	Circadian Locomotor Output Cycles Kaput protein
DNR	dorsal raphe nucleus
GHT	geniculohypothalamic tract
HRT	High responder rat to aerobic training
IGL	intergeniculate leaflet
LRT	Low responder rat to aerobic training
RW	running wheel
SCN	suprachiasmatic nucleus
SED	sedentary
5HT	serotonergic

SISÄLLYS

1 INTRODUCTION

Aerobic exercise extends its effects beyond cardiovascular health. Good aerobic fitness seems to reduce brain tissue loss and reverse negative effects of aging to brain and brain functions (Colcombe et al. 2003; Hillman et al. 2008; Pereira et al. 2006; Van Praag et al. 2005). In rodents, it has been shown that physical exercise increases hippocampal neurogenesis, the formation of new brain cells in hippocampus. Hippocampus is an important brain area for learning and memory (van Praag et al. 2009). Rats with high physical fitness show better learning capacity than rats with low physical fitness (Wikgren et al. 2012). Formerly, it was thought that the production of new neurons, neurogenesis, occurred only during embryonic phase in the mammalian brain (Atlman et al. 1965). However, nowadays it is generally accepted that new neurons are generated throughout life in many species including humans (Erikson et al. 1998; Gage, 2000). New neurons are formed in the olfactory bulb and dentate gyrus of the hippocampus (van Praag, 2009). Many extrinsic and intrinsic genetic factors affect the production on new neurons (Taupin, 2006). Physical exercise is a strong stimulus to enhance neurogenesis in rats (van Praag, 2009; Zhao et al. 2006).

Physical activity certainly has positive effects, but it seems that physical fitness is a stronger predictor of death than physical activity (Karvinen et al. 2015; Myers et al. 2004). When studied with rodents, the result show that rats born with high running capacity, thus with better physical fitness, live longer than rats born with low running capacity even if the low capacity runners are engaged in physical activity (Karvinen et al. 2015).

Genetics that partly determine our physical fitness can be divided to an intrinsic, inherent component and to an extrinsic, acquired component. The inherent component operates in the nontrained state (sedentary) and the acquired component is an adaptive response to physical activity. (Koch et al. 2013.) Like demonstrated in the rat study by Karvinen et al. (2015), low capacity runners were not able to reach the lifespan of the sedentary high capacity runners with physical exercise. This suggests that the innate factor concerning aerobic capacity count the most in longevity. To improve physical fitness and to get the positive effects of physical

exercise, one should do exercise training and engage in physical activity. However, not all individuals get the exact same effects from exercising: variation in the ability to improve exercise capacity exists. Some individuals show large gain (high responders) while others experience no improvement (non-responders) or even negative changes in indices of health. (Bouchard et al. 1999). This is due to the differences in genetic and environmental backgrounds.

To study the training response further Koch et al. (2013) developed a rodent model by two-way artificial selection of rats based on the magnitude of change in running capacity as a result of training. As this selection would go across several generations it would yield an animal model and serve a way to uncover genetic features that are responsible for low and high responsiveness to exercise training. Two lines of rats were produced: low response trainers (LRT) and high response trainers (HRT). Though the two different lines are same in their genotypic level, their differences emerge in the trained stage (Koch et al. 2013). Previous study (Lessard et al. 2013) indicates that LRT rats are more exposed to metabolic dysfunctions and increased adiposity than HRT rats. This results in increased disease risk and might affect LRT rats' longevity. This raises an interesting question: if the LRT rats have greater risk to diseases and they respond badly to physical exercise, do they also have poorer starting point when it comes to neurogenesis?

The aim of this study is to study the circadian differences in spontaneous home cage activity of aged LRT and HRT rats. This is something that has not been studied before. Another and main thing of interest in this thesis is the correlation between the spontaneuous activity, when activity is started in older age and neurogenesis and if any differences between LRT and HRT can be found. This study was made as a part of AFIS (Active, Fit & Smart) -project, in which the main idea is to study how physical activity and aerobic capacity affect and connect with learning and its prerequisites.

2 RATS AS STUDY SUBJECTS

Rats have been used as study subjects for over a century. The laboratory rats, Norwegian rats (Rattus Norwegicus) are excellent choice compared to other animal models in learning and memory studies because the physiological systems involved in these tasks have been more extensively studied in these animals. (Whisshaw et al. 2005.) Rat is also an intelligent animal and it can learn a wider variety of tasks than for example mice. Also, the size of the animal makes it easier to study the organs and their structures. (Iannaccone & Jacob, 2009.) In the following I will discuss more about the circadian rhythm that drives rats' behavior and physiology, their species related nature including spontaneous activity and a special breeding model that has been developed to study the effects of certain genotype.

2.1 Relation between rat age and human age

Laboratory rats live between 2 to 3,5 years (Pass & Freeth, 1993) The life expectancy at birth for a European born in 2015 according to WHO (2016) is around 78 years, females (80,2 years) living approximately eight years longer than male (73,2 years). The relation between rat and human age is not straightforward. Humans and rats have development phases that last for a differing time periods compared between the species. There are also clearl differences in anatomy and physiology. (Sengupta, 2013.) The relation between the ages is represented in table 1.

TABLE 1. Relation between ages (adapted from Sengupta, 2013).

Relation between rat age and human age	
Rat age (years)	**Human age (years)**
6 months (0.5)	18
12 months (1.0)	30
18 months (1.5)	45
24 months (2.0)	60
30 months (2.5)	75
36 moths (3.0)	90

2.2 Circadian rhythm

The main purpose of the circadian clockwork is to determine rhythms in the behavior and physiology. Cycles with a period of around 24 hours are considered to be circadian. Bodily functions like rest and activity cycles, heart rate, blood pressure and urine production are influenced by daily rhythmicity. (Claudel et al. 2007.)

Behavior of mammalian organs follows daily variation that is controlled by an internal circadian timing system, environmental cues, and the interaction between these two. The circadian timing system is composed of a master clock in the brain and subsidiary oscillators in all cells of the body. (Dibner et al. 2010.) In 1972 Stephan and Zucker (1072) localized the master clock, the suprachiasmatic nucleus (SCN) in the hypothalamus in rats. This special area was found by selectively damaging several regions in the hypothalamus. This master clock receives photic (light related) signals from the retina by the retinohypothalamic tract which are transmitted to the neurons in the SCN. SCN receives also nonphotic signals via the geniculohypothalamic tract (GHT) and by serotonergic (5HT) input from the dorsal raphe nucleus (DRN). The SCN in turn influences circadian physiology and behavior via neuronal and humoral pathways and by synchronizing local oscillators in the cells. The cells in peripheral tissues have autonomous and self-sustained oscillators, but the SCN sets the pace

through a variety of pathways discussed above. (Dibner et al. 2010.) The functioning and afferent pathways are illustrated in figure 1.

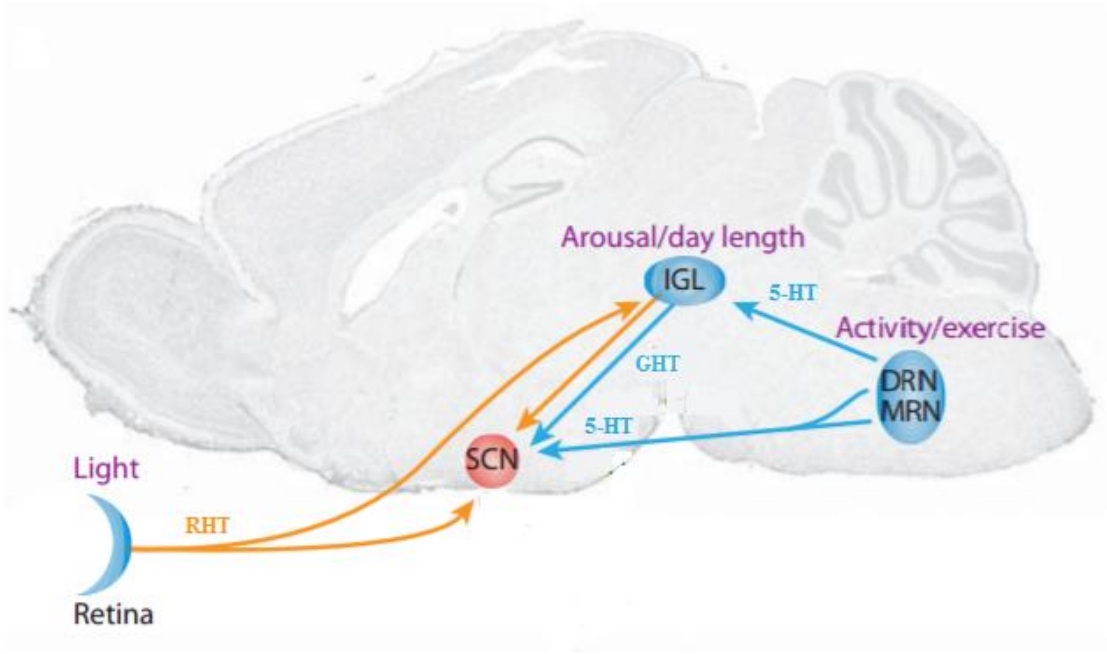


FIGURE 1. Afferent pathways to the SCN in rat. In orange, is presented the photic input and in blue is presented the nonphotic input. Light signals travel to SCN via the retinohypothalamic tract (RHT). The nonphotic input from serotonergic (5HT) and geniculohypothalamic tract (GHT) are transmitted to SCN by dorsal raphe nucleus (DRN) and intergeniculate leaflet (IGL). (Adapted from Dibner et al. 2010.)

At molecular level, there is no difference in the neurons of the circadian clock in SCN and peripheral cells (Balsalobre et al. 1998), but critical difference is in their synchronization. SCN neurons are controlled firstly by the light-dark cues perceived by the retina and peripheral oscillators by chemical cues (Claudel et al. 2007). The integrity of peripheral clocks and the master clock is dependent on several gene activities. The core circadian clock genes are expressed in the SCN and in the peripheral tissues and they form an interconnected

feedback loop. Brain and Muscle ARNT Like 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK) are two proteins which are positive regulators for the transcription of the feedback loop. They act as molecular oscillators which generate circadian rhythm. (Ko & Takahashi, 2006.)

According to studies with rodents, aging affects the circadian rhythm by shortening the circadian period (Morin, 1988), altering the activity rhythm and by decreasing precision of daily activity (Scarbrough, 1997; Valentinuzzi et al. 1997).

Circadian clocks and rhythms have relation with health and wellbeing. Multiple studies demonstrate that circadian disruptions affect physiological processes negatively and might even lead to pathological stages. Epidemiological data shows that shift workers have significantly increased risk of metabolic and cardiovascular diseases and cancer in comparison with general population (Green at al. 2008; Davis et al. 2001). Mutations in CLOCK proteins results in obesity and metabolic syndrome (Turek et al. 2005) and mice with deficiency in BMAL1 protein have impaired circadian rhythm and reduced lifespan. These mice also have symptoms of (for example sarcopenia) premature aging. (Kondratov et al. 2006.)

2.3 Circadian rhythmicity as a determinant of behavior and locomotion in rats

Rats have a circadian rhythm that strongly defines their behavior. Rats are nocturnal animals and for that reason most active at dusk (Whisshaw et al. 2005). In nature, rats spend their time during these hours by digging burrows, finding food, and preparing nests (Armitage, 2004). Circadian rhythm regulates rats' behavior on sleep and wake, feeding and drinking, thermoregulation, reproductive behavior and locomotion; in other words, their activity. Circadian rhythm in turn can be influenced by environmental factors. (Whisshaw et al. 2005.)

The most important timekeeping factors on circadian rhythm are light and feeding. Light exposure promotes sleep and inhibits activity and unfavorable exposure to light can alter rats'

circadian rhythm. If rats have a scheduled time of feeding they usually start to become more active just before the mealtime. (Whisshaw et al. 2005.)

This said, the wheel running of rats, follows a certain daily rhythm – rats are most active and incessant during night time and more active period starts around the time lights are turned off. Another burst in the activity happens when the lights are turned on, but during the bright day time, rats are usually quite inactive. (Suckow et al 2005.) The light-dark cycle guides the daily activity of rats, but they have individual differences related for example to age, and reproductive status. The pattern of running can also vary with individual and between strains. (Whisshaw et al. 2005.) Also, free possibility to run in the running wheel affects circadian rhythm (Yamada et al. 1986).

Many factors affect the activeness of the rats. These include the age, temperature of the environment, feeding (Richter, 1922), stress (Gorka et al. 1996; Harper et al. 1996), social defeat (Meerlo et al. 1996) and estrous cycle (Wang, 1923). The difference in activity between times of day evens out when rats get older (Richter, 1922). According to Richter, it also seems that rats become more nocturnal with increasing age. According to Gorka et al. (1996) chronic mild stress causes disturbances in the circadian rhythms concerning locomotion activity of rats and the decrease in activity is higher in the night-time activity. Stress related decrease in the activity is seen in the mean activity and in the amplitude of the diurnal rhythm.

2.4 Selective breeding model

Both the genetics, also referred to as the genotype, and the environment determine the functioning of our body and organs thus creating an individual phenotype. This applies also to aerobic capacity (Bouchard et al. 1997.) Hence, aerobic capacity can be divided into innate capacity and acquired capacity. Innate capacity is solely determined by genes and therefore independent of training. Acquired capacity is a result of physical exercise and genes that determine one's response to this exercise. (Koch et al. 2001.)

In selective breeding a feature of interest is measured from a larger founder population that consists of a wide heterogenous genetic background. The individuals who demonstrate the extreme values for the measured feature are being bred and at each subsequent generation the new offspring are classified like this based on their phenotype and bred to create the new generation. This process goes on until the change in the feature plateaus. (Whishaw & Kolb, 2005.)

In 1984 the National Institute of Health (NIH) developed a rat model of genetically heterogeneous stock (N:NIH stock). The rats were derived from eight inbred founder strains and the rats were bred for 50 generations using an outbreeding regime to minimize inbreeding. (Hansen et al. 1984.) Later, in 1998 Koch et al. utilized N:NIH stock to create rat lines with different intrinsic aerobic capacities. The motivation for doing this lied in the importance of aerobic capacity in health and diseases. (Koch et al. 2001). The created model presents the intrinsic component of exercise capacity that is seen in nontrained state. After 11 generations of breeding high-capacity runners (HCR) and low-capacity runners (LCR) were formed. (Koch et al. 2001.)

After this, Koch et al. (2013) started to develop another animal model to study the adaptive response to aerobic exercise training. This was based upon the conclusions (Bouchard et al. 1999) that training responsiveness demonstrated a familial inherited heterogenetic pattern. Koch et al. (2013) had a founder population of heterogenous N:NIH rat stock and in each generation the running distance before and after treadmill training period was measured and the difference in the running distance (ΔD) was calculated. They aimed to create rat lines with low response to training (LRT) and high response to training (HRT). After 15 generations of selective breeding the HRT rats improved in their running distance 223 ± 20 m and the running distance for LRT rats declined -65 ± 15 m when given the exact same training environment as presented in figure 2. (Koch et al. 2013).

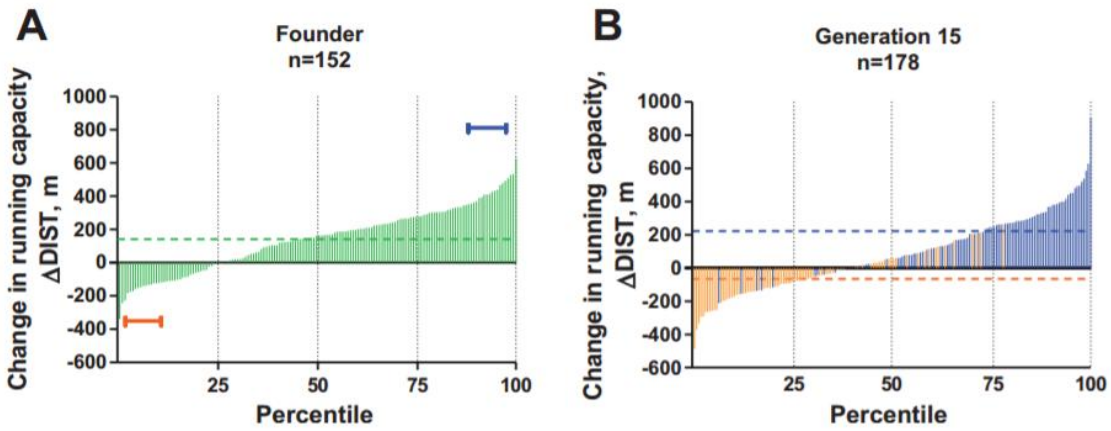


FIGURE 2. Response to aerobic training in genetically heterogenous rat populations. (A) 152 non-selected N:NIH rats put in ascending order depending on the change in the running distance (ΔDIST). The orange brackets indicate the lowest and highest 10th percentile rats that were used as founders to start the selective breeding. (B) Percentile rank score according to the change in running distance for 178 rats after 15 generations arranged from lowest to highest. Light bars indicate LRT rats and dark bars HRT rats. (Koch et al. 2013.)

The LRT and HRT rats have different responses to training but the same intrinsic exercise capacities and body weights. The selected lines had same running distances at both the genetic and phenotypic levels. This means that The HRT and LRT rats have no difference in cardiorespiratory fitness and their exercise capacities are the same in non-trained conditions (Wisloff et al. 2005). The differences between the lines occur due to aerobic training.

It has been examined (Lessard et al. 2013) that the rats born with low exercise response have more metabolic dysfunctions, increased adiposity and impaired exercise-induced angiogenesis. Lessard et al. (2013) concluded from the results that the risk factors are due to increased stress and inflammatory signaling and altered TGF-b signaling – not because of differences in mitochondrial capacity in skeletal muscle. This suggests that LRT rats have lesser exercise-induced plasticity in the muscle (Lessard et al. 2013). However, Marton et al.

(2015) uncovered that LRT and HRT rats had differences in the activity of AMP-activated protein kinase alpha and in certain enzymes that are activated by exercise. This suggests that factors that are associated to mitochondrial biogenesis act differently to aerobic exercise between these rat lines.

3 HIPPOCAMPUS

Hippocampus (Greek for seahorse) is a major component of the limbic system which participates in memory formation and spatial navigation. Hippocampus is situated in the anterior medial region of the temporal lobe. (Kolb et al. 2015, p. 74.) In the following this brain structure is presented including its anatomy and role in memory.

3.1 Anatomy

Hippocampus is a curved brain structure that consist of two hippocampi. Hippocampus is located on the basal medial surface on the temporal lobes and there is one hippocampi on each side of the brain (figure 3). (Kolb et al. 2015, p. 490.)

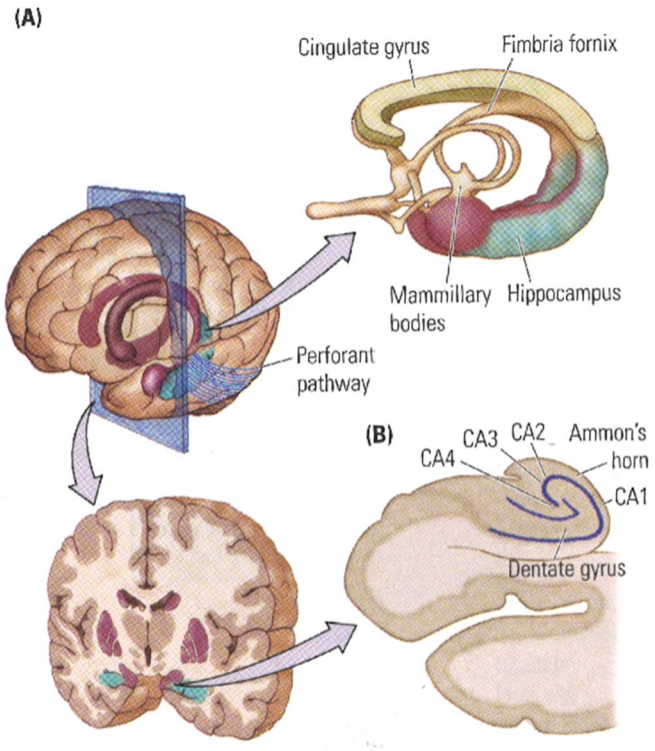


FIGURE 3. (A) Hippocampus is situated in the temporal lobe, one hippocampi in each side of the brain. It's connected to other brain structures with perforant pathway and with fimbria fornix. (B)

Hippocampus has two structures with different kind of cells, the Ammon's horn and the dentate gyrus. (Kolb et al. 2015, p. 490.)

Hippocampus is connected to other parts of the brain through perforant pathway and fimbria fornix. Perforant pathway connects hippocampus to the posterior temporal cortex and fimbria fornix to the thalamus, prefrontal cortex, basal ganglia and hypothalamus. Hippocampus is a structure that separates the posterior and frontal neocortex. Input from neocortex go to Ammon's horn via the dentate gyrus. (Kolb et al. 2015, p. 491.)

Hippocampus has two components that differ by their cell types: cornu ammonis and dentate gyrus. Cornu ammonis can be further divided into CA1, CA2, CA3 and C subfields and contains pyramidal neurons. Dentate gyrus is made of granule cells. In hippocampus, the pyramidal cells act as motor cells and granular cells as the sensory cells. (Kolb et al. 2015, p. 490.) Neurogenesis takes place in the dentate gyrus in the hilus and mostly in granule cell layer (Kolb et al. 2015, p. 705). More specific overview to the cell structure is presented in figure 4.

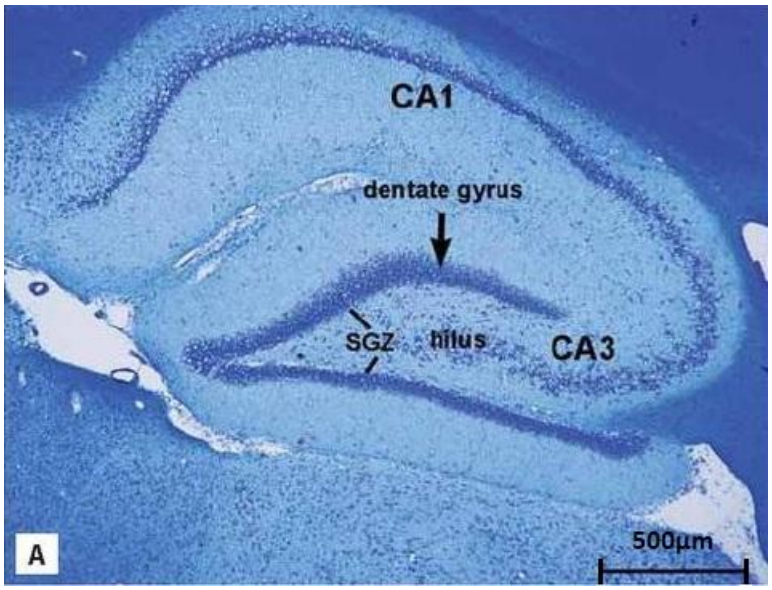


FIGURE 4. Rat's hippocampus stained with cresyl violet. Two regions: the dentate gyrus and cornu ammonis with its sub regions CA1 and CA3 are represented. The subgranular zone (SGZ) stands out with darker blue. (Adapted from Bálentová et al. 2015)

3.2 Role in memory

Based on cases of patients with damage in the hippocampus or in the neural hippocampal connections, it has been seen that hippocampus has an important role in memory. The role is special in episodic memory that accounts for memories made of personal experiences. (Kolb et al. 2015, p. 492.) Hippocampus might also have a role in the function of creating new memories, but the actual memories are retained in some other part of the brain. This has been concluded since old memories can be retrieved after damage in hippocampus, but forming new memories is not possible. Still, patients with amnesia can learn new motor skills that indicate that not all learning requires the hippocampus. (Nolte, 2002.) It seems that the memory system is rather complicated and multiple memory systems are working in sync.

Other important role of hippocampus is in spatial memory, learning spatial information like locations of certain objects (Kolb et al. 2015, p.492). Hippocampus is seen to be a mediator of spatial navigation and spatial memory (Epp et al. 2010). The results of studies made with rats

with lesions in hippocampus, showed that rats with the lesions had impairments in retention of preoperatively learned spatial information and they forgot spatial information. (Jarrard, 1995.) Hippocampus has “place cells” that are activated in specific locations within an environment which helps to form a spatial representation on the environment (O’Keefe et al. 1971; O’Keefe, 1976). Hence, the role of the hippocampus and its functions are still in many ways only assumptions.

4 BRAIN HEALTH AND HIPPOCAMPUS RELATED FACTORS

Physical activity has many beneficial effects on brain. This includes neurogenesis (Eadie et al. 2005; Zhao et al. 2006; Van Praag et al. 2005). Another key factor that affects our brain health is genes. Genes are also related to the benefits that physical activity provokes (Marton et al. 2016; Nokia et al. 2016). In the following the benefits of physical activity and the role of genetic factors in respect to physical exercising are discussed.

4.1 Physical activity and circadian rhythmicity

Physical exercise has many beneficial effects on brain and on its structures and functions. Exercise has a clear role in reducing atherosclerotic cerebrovascular diseases (Knopman et al. 2010) by for example increasing blood capillary growth in the motor area of cerebral cortex and by enhancing blood flow in these areas (Swain et al. 2003). However, it seems that the positive effects of aerobic exercise are covering more than just the ones of cardiovascular and cerebrovascular health. Adults who have high cardiovascular fitness show significantly higher activation in cortical regions when studied with functional magnetic resonance imaging (fMRI) (Colcombe et al. 2004). Barnes et al (2003) did a follow-up study with 349 individuals who were over 55 years old. In six years, the ones who had the worse cardiorespiratory fitness at baseline had greater decline in their cognitive functions. This indicates that high cardiorespiratory fitness may protect against cognitive degradations in older age. Kramer et al. (1999) did study where previously sedentary elderly persons between the age of 60 to 75 years did either aerobic or anaerobic exercise for six months. The results showed that the ones who took part in aerobic training showed improvements in tasks that required cognitive skills (Kramer et al. 1999).

But what happens in the brain due to exercise? Exercise seems to affect the actual brain mass. Brain volume and cognitive abilities are decreased by aging (Smith et al. 2000), but good cardiovascular fitness spares from these. The regions in the brain which are most affected by aging (tissues in frontal, parietal and temporal cortices) seem to benefit the most from exercise. (Colcombe et al. 2003.) The brain volume protective effects are seen to cover the

hippocampal areas also. Erickson et al. (2011) performed a one-year randomized controlled trial with 120 seniors (55-80 years old) and discovered that hippocampal volume increased by 2 % in aerobic exercise group. In the control group, hippocampal volume decreased, but individuals who had higher physical fitness level had milder changes. The increased hippocampal volume was reflected to improvements in memory functioning, indicating that aerobic exercise has neuroprotective effects. According to this study, it seems that even if physical exercise is started in older age, it might have potential for augmenting brain volume and cognition. (Erickson et al. 2011.) In a long-term cohort study (Erickson et al. 2010) with healthy adults it was noted that weekly physical activity (walking) was positively associated with hippocampal volume nine years later. Higher amount of walking was associated with a lower risk of cognitive impairments. (Erickson et al. 2010.)

Exercise increases the amount of neurogenesis (Eadie et al. 2005; Zhao et al. 2006; Van Praag et al. 2005), but the reason which leads to this is not clear. Studies (Steiner et al. 2008; Olson et al. 2006) report that exercise induces the proliferation of neurons, even if the net amount of neurogenesis is not increased. It might be that more cells are matured to neurons (van Praag et al. 1999; van Praag et al. 2005). According to Fischer et al. (2014), physical exercise increases AHN because the number of surviving neuronal precursor cells increases, not by lengthening the lifecycle of the cells. Van Praag et al. (2005) also suggested that the properties of new neurons do not change with aging. Rather due to exercise the hippocampal environment of the dentate gyrus can sustain neurogenesis. Contrary, a study by Eadie et al. (2005) shows that the dendrites of neurons in dentate gyrus are changed morphologically when exercising is performed. The length and complexity of the dendrites and their spine density was increased. Zhao et al. (2006) did not see any changes in the spine densities of new neurons in the brains of voluntary running mice. Mice which run in the wheels had significantly higher number of newborn neurons than mice in the control group. It also seems that physical exercise increases the incorporation of new-born hippocampal cells and their survival (Lee et al. 2013).

Exercise also seems to affect the proteins and enzymes in hippocampus. Wheel running increased levels of brain-derived neurotrophic factor (BDNF) (Adlard et al. 2004; Farmer et al. 2004). BDNF promotes neuronal survival and regeneration and hence has an important

role in neurogenesis (Thoenen, 1991). Fordyce and Wehner (1993) discovered that physical activity enhanced the activity of hippocampal protein kinase C, which might be an important enzyme in learning.

The positive effects of exercising are reflected to cognitive skills. Van Praag et al. (2005) noticed that physical activity generated a 2 to 12 -fold increase in spatial learning performance in physically active mice with enhanced hippocampal neurogenesis. The improvements are seen in spatial learning (Berchtold et al 2010; van Praag et al. 1999; van Praag et al. 2005). It even seems that the cognitive benefits of physical exercise are sustained over several weeks after the exercising has ended and this might mean that the effects of the exercise continue to evolve in the brain for a period (Berchtold et al. 2010). Van Praag et al. (2005) studied the effects of voluntary wheel-running on young and aged mice. After one month of training, the aged runners showed faster acquisition and better retention of the right route on a maze test than their sedentary controls. Also, in the aged runners group, the amount of cell survival was returned to the level of the young controls.

Interestingly, Holmes et al. (2004) demonstrated in their study with mice, that the effect of exercise on adult hippocampal neurogenesis is dependent on not only on the amount of the exercise but also on the circadian phase. Exercise done during some specific circadian phases can further add neurogenesis. Circadian rhythm has also effects on its own. Circadian rhythm influences brain related functions, for example learning, cognitive functions and memory formation (Gerstner et al. 2009; Gerstner, 2010). As stated earlier, disfunctions in circadian rhythm or in the circadian proteins have negative, even pathological effects (Green at al. 2008; Davis et al. 2001; Kondratov et al. 2006; Turek et al. 2005).

According to a study by Bouchard-Cannon et al. (2013) it seems that circadian clock regulates adult hippocampal neurogenesis. Circadian rhythm allows the neural progenitor cells to enter the cell cycle to be proliferated into granule cells by establishing a temporal window. Circadian clock also limits the number of neural progenitor cells to enter the cell cycle and restricts the number of cell divisions. BMAL1 proteins seems to be essential for timing the

neural progenitor cells to enter the cell-cycle and suppressing the number of cells to go through proliferation. (Bouchard-Cannon et al. 2013.)

4.2 Genetic factors

The relation between AHN and exercise has been mainly studied concerning running (van Praag et al. 1999; van Praag et al. 2005). Since aerobic fitness is beneficial for the brain, and has functional and structural effects on it and that aerobic fitness can be partly improved by training, it might be that one factor that influences the brain is the inherited ability to increase aerobic capacity – the responsiveness to exercise.

Nokia et al. (2016) studied the effects of aerobic training, high intensity training (HIT) and resistance training to LRT and HRT rats for promoting AHN. They found out that AHN was more present in HRT rats in response to voluntary aerobic wheel running. The results indicate that rats that have been selectively bred for high response to aerobic exercise indeed show greater AHN (figure 7). No statistically significant results were found between HIT or resistance training and AHN. The positive result of HIT training to AHN might have been outlined by the negative effects of stress caused by forced treadmill training. (Nokia et al. 2016.) According to a master's thesis (Harri & Heiskanen, 2014) done to the same study project (Metapredict) hippocampal neurogenesis is not dependent on rat line. The effects of aerobic and resistance training to AHN in HRT an LRT rats was examined. Aerobic exercise was more efficient to promote neurogenesis but not differences between HRT and LRT rats were found.

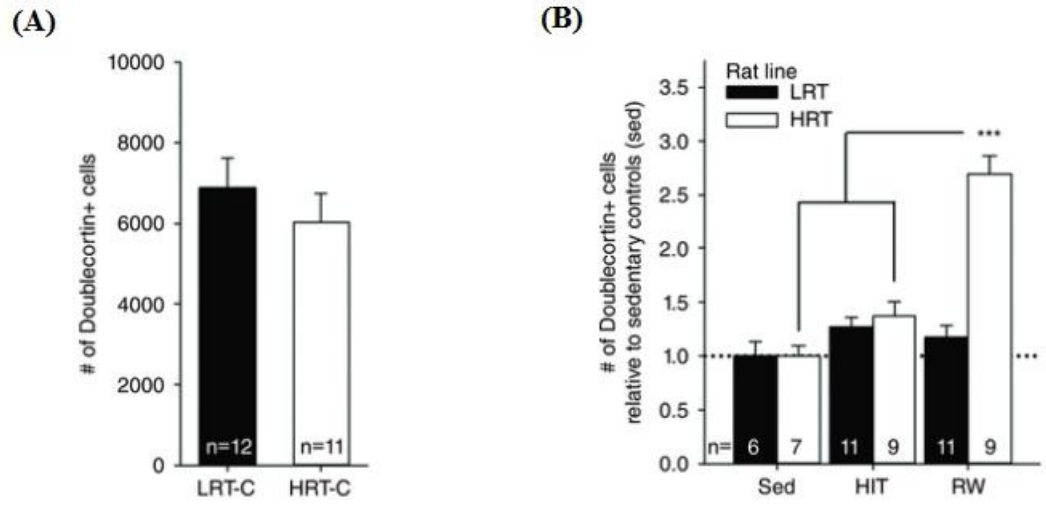


FIGURE 5. (A) No difference in adult hippocampal neurogenesis (AHN) the baseline in the two rat lines. (B) HRT rats in voluntary wheel-running group had more new neurons in hippocampus than their sedentary counterparts or HRT rats in the HIT group (*** $p<0.001$) (Nokia et al. 2016).

Marton et al. (2016) studied the effects of aerobic training on LRT and HRT rats with the purpose of testing whether the genetic difference for trainability affects hippocampal brain functions. Their study reveals that the aerobic trainability has an influence on the brain functions of rats studied and the differences emerge in the trained state (figure 8). According to their study HRT rats which were aerobically trained showed better spatial learning abilities and cognitive flexibility. (Marton et al. 2016.)

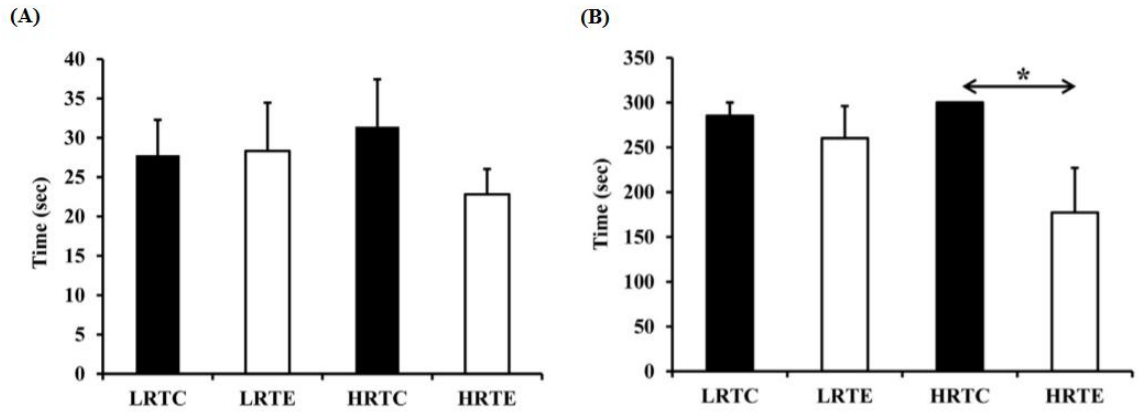


FIGURE 6. Cognitive functions pre-(LRTC and HRTC) and post-training (LRTE and HRTE). (A) Reverse Morris maze test and (B) passive avoidance test scores to assess brain function. In passive avoidance test, HRTC rats had perfect performance which was decreased after training ($p<0.05$). Groups are as follows: Low response trainers control (LRTC, n=6), exercised LRT (LRTE)(n=7), high response trainers control (HRTC, n=6) and exercised HRT (HRTE, n=8) groups. The bars represent the time in which the animals entered the "safe, dark chamber" (Marton et al. 2016).

Their (Marton et al. 2016) other key finding was that the responses to exercise were different in the levels of markers of apoptosis, a process of programmed cell death. The ratio of Bax/Bcl -2 decreased in HRT rats, but increased in LRT when they did exercise (figure 9). This means that when LRT rats were participating in exercise more apoptosis related markers were measured. (Marton et al. 2016.)

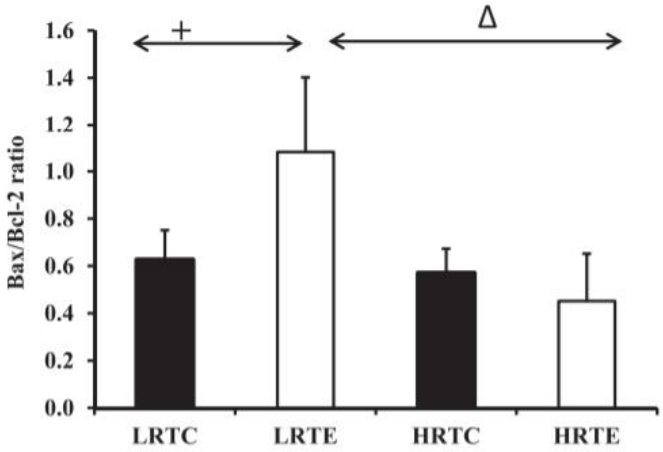


FIGURE 7. Low response trainers (LRT) rats had elevated apoptosis levels after exercising, compared to high response trainers (HRT). Apoptosis is determined by the ratio of Bax and Bcl-2 (Marton et al. 2016). Groups are as follows: Low response trainers control (LRTC, n=6), exercised LRT (LRTE, n=7), high response trainers control (HRTC) (n=6) and exercised HRT (HRTE) (n=8) groups. The bars represent the time in which the animals entered the "safe, dark chamber" (Marton et al. 2016).

4.3 Formation of new hippocampal neurons

Although, for a long time it was believed that the adult brain cannot produce new neurons (Altman et al. 1965), it is now generally accepted that new neurons are produced true life (Erikson et al. 1998; Gage, 2000) - even in the human brain (Eriksson et al. 1998). Adult neurogenesis happens in certain areas of the brain: in subventricular zone, in the olfactory bulb and in the dentate gyrus (Kolb et al. 2015). When discussed about the neurogenesis in hippocampus in adult life, a term adult hippocampal neurogenesis (AHN) is used (Kempermann, 2011; Nokia et al. 2016; van Praag et al. 1999: van Praag et al. 2005).

The amount of newly produced neurons correlates with better results in tasks depending on learning (van Praag et al. 1999; van Praag et al. 2005). When new neurons are created, the number of synapses is increased (Zhao et al. 2006). Based on these, it seems that AHN and certain kind of learning are in connection. AHN is a complex process and still in many ways unknown.

Adult hippocampal neurogenesis takes mainly place in the dentate gyrus in hippocampus. First neural progenitor cells are proliferated in the subgranular zone of dentate gyrus. Most of the proliferated cells will differentiate to be dentate granule cells and a small portion will proliferate to be glia cells. The newly born dentate gyrus cells have to go through a long process of about eight weeks before they have morphological and physiological properties like mature cells. (Deng & Aimone, 2010.) This process is illustrated in figure 5.

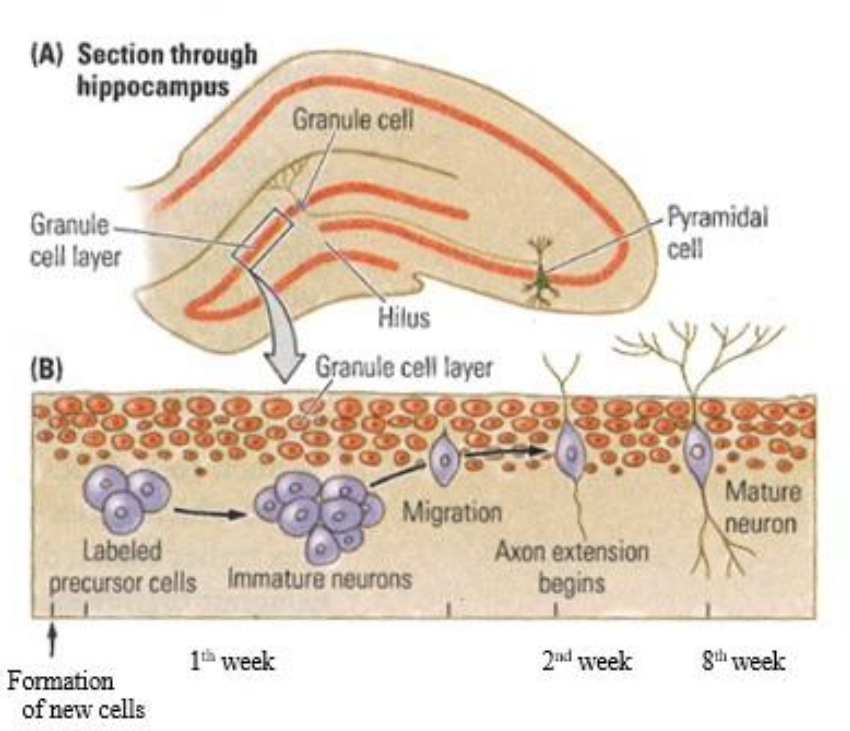


FIGURE 8. Neurogenesis in hippocampus. (A) Cross section of hippocampus which demonstrates the granule cell layer and hilus where neurogenesis takes place. (B) Process where precursor cells differentiate into immature neurons, migrate to appropriate location and develop to mature neurons to form mature connections. (Adapted from Kolb et al. 2015, p. 705.)

During the first week, the cells have initial differentiation phase and they migrate into the inner granule cell layer of the dentate gyrus. Here the cells extend their cellular processes but are not synaptically integrated to the neural network. (Esposito, 2005; Zhao et al. 2006.)

During second week the cells start to become more neuron-like and began to grow dendrites which extend towards the molecular layer. The axons start to grow through the hilus towards the CA3. (Zhao et al. 2006.) The afferent and efferent connections to the local network start to form during the third week. The spines of these new cells become to merge with the dendrites of adult born dentate gyrus cells and form synapses with the axon fibres in the perforant pathway. (Zhao et al. 2006; Toni et al. 2007.) New dentate gyrus cells still have some characteristics concerning their excitability properties which differentiate them from mature cells. During next weeks the cells mature by their physiological and connectivity properties and around week eight they become intrinsic from their mature counterparts. (Esposito, 2005; Zhao et al. 2006.)

AHN is a continuous process in which cells proliferate into granule cells and become incorporated into hippocampal neuronal networks (Aimone et al. 2014). The mature neural cells in hippocampus interact with each other and with other brain structures (see figure 6).

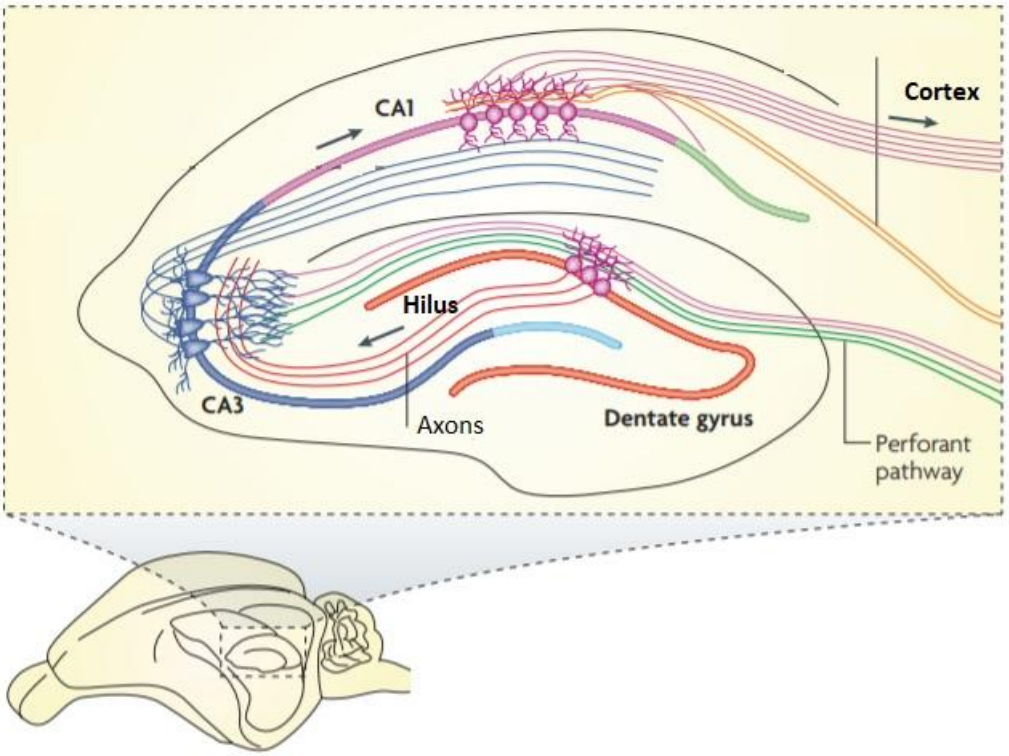


FIGURE 9. Neural connections in the hippocampus of a rodent. The cells born in the dentate gurys extend their axons towards the CA3. Dentate gyrus sends projections to the pyramidal cells in CA3 through these axons. The information is further transmitted to CA1 neurons and from there to the cortex. (Adapted from Deng & Aimone, 2010.)

Many factors seem to affect the neuronal proliferation and survival. Aging and stress seem to decrease both events. (Kolb et al. 2015, p. 706.) Vice versa, e. g. antidepressants that stimulate serotonin production, or physical activity and learning tasks seem to increase neuronal proliferation and survival. (Muotri et al. 2009; Snuder et al. 2009; Van Praag et al. 1999; van Praag et al. 2005.)

5 AIMS OF THE STUDY

The aim of this study was to examine the role of spontaneous activity on brain health when the activity is started in older age. In this thesis, with spontaneous activity it is referred to the vertical locomotion of the rats around their cage and running in the running wheel. The rats represented two lines (HRT and LRT) which were selectively bred to have difference in their inherent responsiveness to physical exercise done at young age. The objective was to examine the spontaneous activity of HRT and LRT rats and its effects on adult hippocampal neurogenesis and possible differences in neurogenesis between these two lines. The spontaneous activity of HRT an LRT rats has not been examined before.

The research questions of this study are:

1. Are there differences or trends in spontaneous activity between HRT and LRT rats?
2. Does voluntary physical exercise affect adult hippocampal neurogenesis when started at older age?
3. Are there differences between LRT and HRT rats in the amount of neurogenesis when exercising is started in older age?

From these research questions we hypothesize the following:

1. HRT rats are more spontaneously active than LRT rats.
2. Rats in running wheel group (RW) are spontaneously more active than rats in sedentary control group (SED) (Karvinen et al. 2015; Novak et al. 2012).
3. Voluntary exercise has positive effects on brain although exercising is started on older age (Kramer et al 1999; Lee et al. 2013; van Praag et al. 2005).
4. Neurogenesis is more present in HRT rats compared to LRT rats (Nokia et al. 2016).

6 METHODS

In this chapter the methods used in this study are presented. This includes the reporting of the subjects, protocol, data collection using ground reaction force plates, sampling of the collected data and the statistical methods.

6.1 Animals

The rats used in the study were genetically heterogenous N:NIH rats, both males and females. These rats represented the 21th generation. The phenotyping of the rats was already done in Michigan from where the rats were flown to Jyväskylä for the research at the end of April 2016. The rats were rather old when the study begun. The mean age was 1.6 years and considering the findings of Sengupta (2013), the age of these rats represented roughly the human age of 45 years.

Both HRT and LRT rats were included in the study. After one week of being accustomed to their new environment and being handled, the rats were tested with a range of physiological and behavioural tests. Based on these tests, the rats were divided into groups of wheel runners and sedentary. Individuals which seemed to be more active were placed in the RW group and vice versa. The grouping was done in June.

All together 79 rats were included in the study. Of the 75 rats, 39 were females and 40 males. In the group of females, 20 were HRT and 19 LRT and 16 belonged to sedentary group and 23 to running wheel group. In the group of male rats 20 were HRT and 20 were LRT rats, and 16 belonged to sedentary group and 24 to running wheel group. Outcome of the grouping presented in table 2.

TABLE 2. Stratification of rats into high running (HRT) and low (LRT) lines by exercise group and sex.

	HRT			LRT		
Sex	Male	Female	All	Male	Female	All
Total (n)	20	20	40	20	19	39
RW (n)	12	12	24	12	11	23
SED (n)	8	8	16	8	8	16

This research was part of a larger study. The same rats were tested with numerous behavioral tests such as open field and Barnes maze tests, startle reflex in prepulse-inhibition test and body composition measurements. These experiments are not considered in this thesis.

6.2 Protocol

The study protocols were approved by the Animal Care and Use Committee of the Southern Finland and the tests done to the rats in the U.S. were approved by the Animal Care and Use Committee at the University of Michigan. Experiments were done in accordance with the guidelines of the European Community Council directives and European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

The rats were kept in single-house cages (size 480 x 267 x 470 mm with a running wheel and 480 x 375 x 210 mm without a wheel (Tecniplast S.p.A., Buguggiate, Italy). Cages were kept in cage-rack, 24 cages in a rack. The rats in the running wheel group and rats in sedentary group were held in separate rooms. The rooms were air-conditioned with the ambient temperature kept at 21 ± 2°C and the relative humidity at 55 ± 10 %. With artificial lightning, a light cycle of 12 h light and 12 h darkness was provided. The lights were turned off at 8 pm and turned on at 8 am. The cages were equipped with aspen chips as a base, wooden nesting material (Tapvei Oy, Kaavi, Finland) and with a plastic tube to enrich the environment. The rats were fed with pellet food (R36, Labfor/Lantmännen, Malmö, Sweden) and the energy

content was calculated. Water and food was provided for the rats ad libitum. The weight and eating of the rats was monitored every other week and ethical instructions were followed throughout the research.

Before the actual study started the rats were familiarized to being handled for a week. During the study, the runner rats had running wheels in their cage. LRT rats could run as much as they wanted to, but HRT rats had breaks in their wheels and they were allowed to run daily the equal distance as their LRT counterparts. Whole intervention lasted about 9 weeks.

The spontaneous activity measurements were done on several days using ground reaction force plates. There were 23 different measurement points between 29th of June and 16th of September. Usually the spontaneous activity was measured from six rats simultaneously, so that each rat was in its own cage that was places over a ground reaction force plate. It is of note that during the spontaneous activity measurements rats were allowed to run voluntarily, without breaks. The spontaneous activity was measured for 46 - 70 hours nonstop. Each rat had at least two measurements of spontaneous activity, but some rats went through three measurements. The necropsies were done between the 5th of September and 5th of October. The mean age of the rats at the time of necropsy was 1.9700 ± 0.004 years. Study protocol is presented in figure 10.

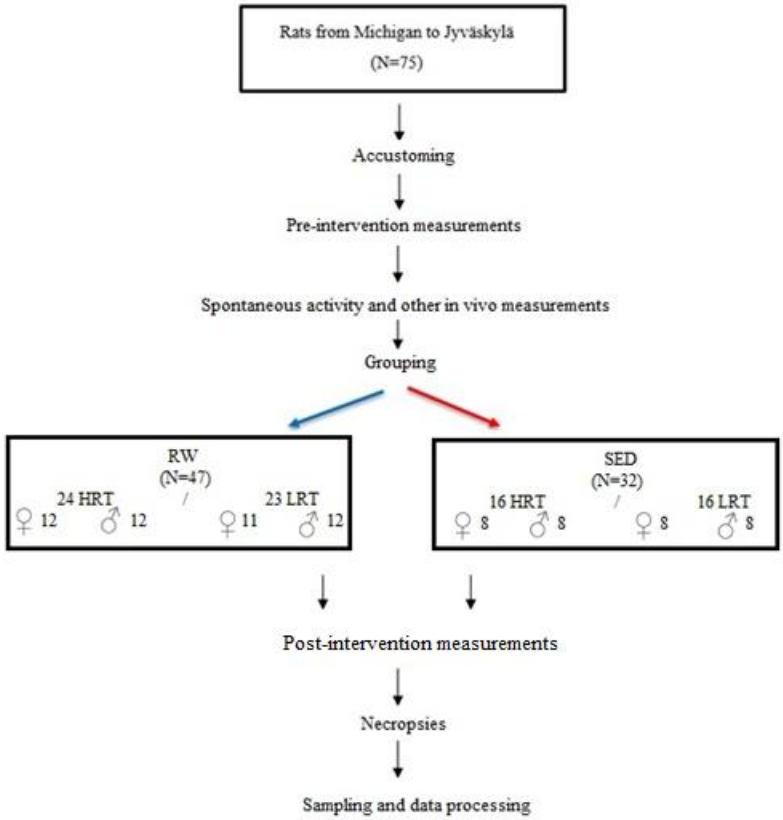


FIGURE 10. Protocol presented in illustration. The duration of the intervention was 14 weeks.

6.3 Data collection – ground reaction force plates

To measure the spontaneous activity of the rats, a ground reaction force plate method was used. This measurement system was developed in the laboratory of the Faculty of Sport and Health Sciences (by Mika Silvennoinen, Timo Rantalainen and technical staff). The use of ground reaction force plates to measure the activity of rodents has been validated by Silvennoinen et al. (2014). Also, according to Rantalainen et al. (2011), there are no differences in the measured activity when using videotaping method or ground reaction force plates.

The force plates were placed on their own shelves with rubber paddings between the pedestals and ground to avoid crosstalk between the force plates. To stabilize the base the four force sensors were attached on a stainless-steel board (730 x 300 x 30 mm), forming a rectangular.

The sensor consisted of bridge formed by stainless steel sheet fixed from both of its ends. There were two strain gauges (1-LY41-6/700, HBM, Darmstadt, Germany) attached on both sides (upper and lower surface) of the sheet. These four strain gauges were connected to form Wheatstone bridge. The output voltage from the sensor is directly related to the vertical force bending the steel sheet and attached strain gauges. The forces were mediated by a pin (diameter 4 mm) that was attached on the top of the steel sheet in the middle of strain gauges. The pins of the four sensors formed a rectangle (500 x 275 mm). The pins supported a glass plate (670 x 310 x 4 mm). To prevent horizontal movement of the glass plate, rubbery ring adapters were attached under the plate. When the home cage activities of rats were measured, the cage was put on top of the glass plate. The vertical movements of the rats induced reaction forces that were detected as voltage output changes. The output voltages from the sensors were pre-amplified (AD620, Analog Devices Inc.,Norwood, MA,USA) before the data was collected. The amplification in each sensor was adjusted to 0.1 kg/V. The signal passed through a controller unit with a capability to zero all sensors. The digitizing of the data was done by 14-bit A/D converter (DI-710, DATAQ Instruments, Akron, Ohio, USA) with a sampling rate of 20 Hz. A measurement range of ±500 g was used, with the digitization precision of <0.06 g. (Silvennoinen, 2016.)

From the collected activity data, an activity index was calculated. This kind of activity index was developed by Biesiadecki et al. (1999) to measure the activity of rats. To get the activity index, firstly the force from all the four sensors was summed The summed signal was smoothed by 10 Hz zero lag low pass Butterworth filter. From this data, the activity index was calculated. The means of the absolute values of the differences between consecutive force values were calculated for every second (from 20 values per second). The one second means were divided by the body mass (kg) of the measured rat to obtain a single body mass normalized value for spontaneous activity. Lowest activity index value from a 5-minute interval, so called background activity, was analyzed from the collected data and this value was scaled to the whole measurement time. The value was used as an estimate of the amount

of inactivity representing forces caused by an inactive rat (e.g. heart beat and breathing) and measurement noise. Finally, the contribution of background activity was subtracted from the activity indexes, and resulting values were used to describe the spontaneous vertical activity of the measured rats. (Silvennoinen, 2016.)

6.4 Data sampling

The data collected using the ground reaction force plates were first examined with WinDaq Waveform Browser 2.95 (DATAQ Instruments, Akron, Ohio, USA). With this program the activity data was checked manually to exclude incorrect data. Inaccurate data was usually caused by malfunctions in the ground reaction force plates. Example of the collected data viewed with WinDaq in figure 11.

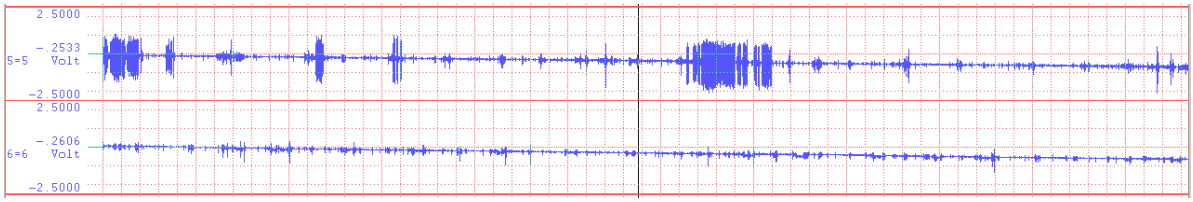


FIGURE 11. Example of the spontaneous activity data examined with WinDaq Waveform Browser. The picture presents data of two rats from two different ground reaction force plates.

After the manual check, the data was analyzed with MatLab 9.1 (MathWorks) to get minute by minute activity index from the collected data. If any errors were detected in the manual examination, this data was excluded in this phase. The data was then collected to an excel file, in which the activity indexes were calculated to hourly means and further to mean values in different periods of the day. The analysis periods were morning (6 am -10 am), day (11 am- 5 pm), evening (6 pm-10 pm) and night (11 pm – 5 am).

For the neurogenesis analyses, the rats were euthanized, the brains collected and the hemispheres and cerebellum separated. Right hemisphere was fixed in 4% paraformaldehyde,

washed with 0.1 M PBS and cryoprotected in 30% sucrose solution. Fixed brain tissue was serially sectioned to 40 µm free-floating slices with sliding microtome (Leica SM2010). Every 12th section was put in the same eppendorf tube, the slices were preserved in cryoprotectant solution and frozen (-20ºC). Every tube contained a comprehensive sample of about 10 slices (with 480 µm distance between each other) from the whole hippocampus. New neurons were stained with a primary antibody for Doublecortin (sc-0866, Santa Cruz Biotechnology). In the immunostaining, secondary antibody was Biotinylated Rabbit Anti-Goat IgG (BA-500, Vector Labs/Mediq), and tertiary Streptavidin-Horseradish Peroxidase Conjugate (RPN1231, GE Healthcare/VWR, USA). Immunostaining was visualized by using diaminobenzidine (DAB) as a chromogen. After Doublecortin immunostaining, free-floating sections were mounted on the object glass and Cresyl Violet was used to counterstain the sections. Immunopositive cells had a brown label and they were counted with a light microscope (Zeiss, Jena, Germany) using 20x objective the granular cell layer and in the hilus on dentate gyrus. The total number of new neurons in the whole hippocampus was calculated by multiplying the counted number by 12 (every 12th section was calculated) and by 2 (only one hemisphere was stained). This number represented the amount of neurogenesis in this thesis.

6.5 Statistics

All of the data were analyzed using SPSS Statistics software (version 22, IBM, USA). Statistical significance was set at $p<0.05$ and high significance at $p<0.001$. The normality was tested by using the Shapiro-Wilk's test. Because the sample sizes were small, and the spontaneous activity indexes were not normally distributed, nonparametric tests were used when spontaneous activity was analyzed. The spontaneous activity between different times of day was tested by using Related-Samples Friedman's Two-Way test. The differences in the spontaneous activity between lines, sexes and groups were tested with the Independent Samples' Mann-Whitney U-test.

The normality of neurogenesis was tested with Shapiro-Wilk -test. Because the sample sizes were small and not normally distributed, nonparametric tests were used. To test the

differences of neurogenesis in between groups, lines and sexes the Independent Samples Mann-Whitney U-test was used and Kruskal-Wallis test when more than two groups where compared.

Two-tailed Kendall's and Spearman's correlations were run for the neurogenesis and spontaneous activity parameters, respectively. These statistical tests were also used to test the correlation between running amount and spontaneous activity.

7 RESULTS

There were no significant differences in the daily average spontaneous activity of a rat between measurement days ($p>0.500$, N=43). Therefore, if a rat had more than one measurement day, an average spontaneous activity in a given time of day (morning, day, evening and night) was used in the final analysis.

7.1 Spontaneous activity

The range of spontaneous activity index was 0.019 – 0.317 and the mean between all subjects was 0.051 ± 0.005 (N=73). The mean of the activity index in the group of HRT female rats was 0.082 ± 0.016 (N=18) and in LRT female rats 0.049 ± 0.005 (N=17). In the group of HRT male rats the mean activity index was 0.034 ± 0.003 (N=18) and in LRT male rats 0.030 ± 0.001 (N=17).

The mean values of the activity indexes of HRT and LRT rats according to sex and study group and according to time of the day are presented in table 3. The correlations and statistical differences concerning the activity index measurements are presented in the upcoming chapters (7.1.1 – 7.1.5).

TABLE 3. Activity indexes in HRT and LRT rats according to sex and study group. The daily average activity index and activity index in every measurement period (morning, day, night and night) are presented in the table.

	HRT				LRT			
Sex	Male		Female		Male		Female	
Group	RW	SED	RW	SED	RW	SED	RW	SED
ActivityIndex (mean)								
Time of the day								
Morning	0.029	0.029	0.130	0.041	0.028	0.026	0.044	0.031
Day	0.021	0.021	0.073	0.036	0.019	0.023	0.022	0.025
Evening	0.063	0.043	0.166	0.056	0.042	0.040	0.095	0.043
Night	0.034	0.028	0.107	0.037	0.031	0.028	0.064	0.031
Daily average	0.037	0.031	0.121	0.045	0.031	0.030	0.059	0.033

7.1.1 Correlation with wheel running

The activity indexes in this study represent the rats' movement in their cages. Both, the moving around in the cage and running in running wheel are represented in the collected activity. There was a statistically highly significant positive correlation between the average distance run per day and the average spontaneous activity ($r=0.760$, $p<0.05$, $N=42$). From the data, it's not possible separate what amount of the activity stems from running in the running wheel. When the correlation was separately run for both sexes, a statistically significant positive correlation was only seen in female rats ($r=0.626$, $p<0.05$, $N=22$). Because the rats in sedentary group did not have running wheels, the correlation was only run to rats in running wheel group. Correlation is presented in scatterplot in figure 12.

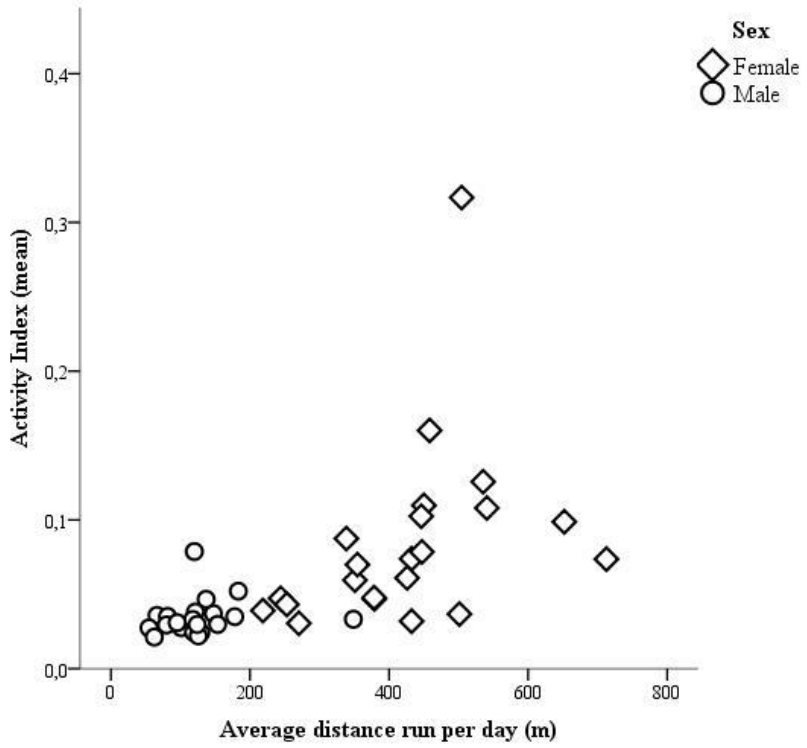


FIGURE 12. Correlation between spontaneous activity and wheel running. Statistically significant correlation ($r = 0.760$, $p<0.05$) was found in the group of female rats. No correlation was seen in the group of male rats.

7.1.2 Circadian rhythm

The rats were most active during evening (6 pm – 10 pm) and most inactive during the daytime (11 am – 5 pm). The amount on activity was similar during night (11 pm – 5 am) and in the morning (6 am – 10 am). The mean values of the activity indexes are presented in the table 4.

TABLE 1. Means and standard deviations of measured activity indices of female, male and rats all together.

	Spontaneuous Activity Index		
Time of the day	**Female**	**Male**	**All**
Evening	0,091 ± 0,067	0,047 ±0,028	0,070 ±0,055
Night	0,063 ±0,048	0,030 ±0,011	0,047 ±0,039
Morning	0,063 ±0,088	0,028 ±0,009	0,046 ±0,065
Day	0,039 ±0,060	0,020 ±0,005	0,030 ±0,044

The difference in spontaneuous activity due to the time of the day was statistically highly significant between all the times of day when pairwise comparison was done, except between night and morning. These findings are presented in figure 13.

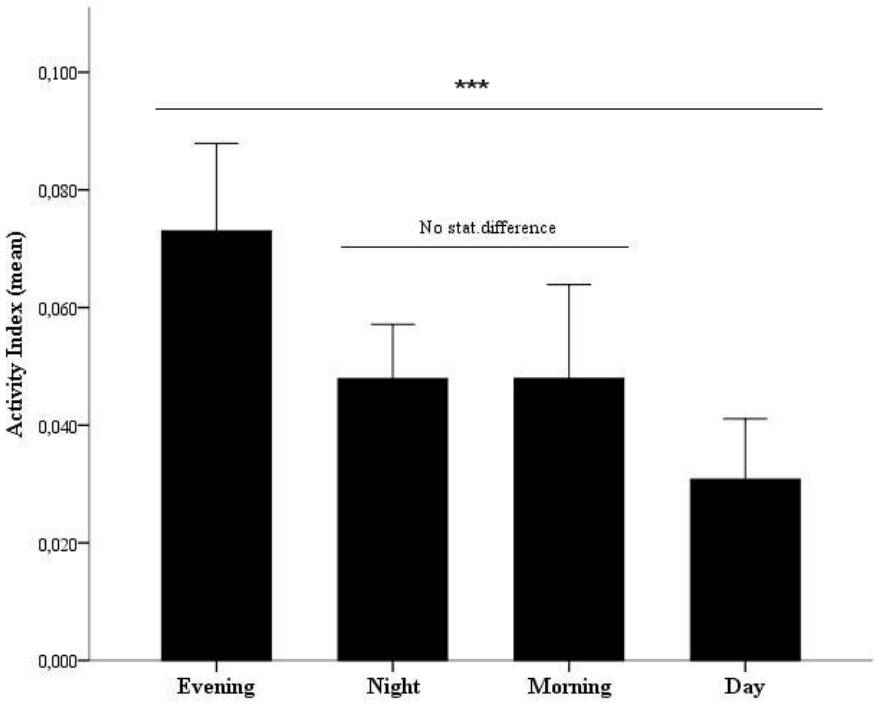
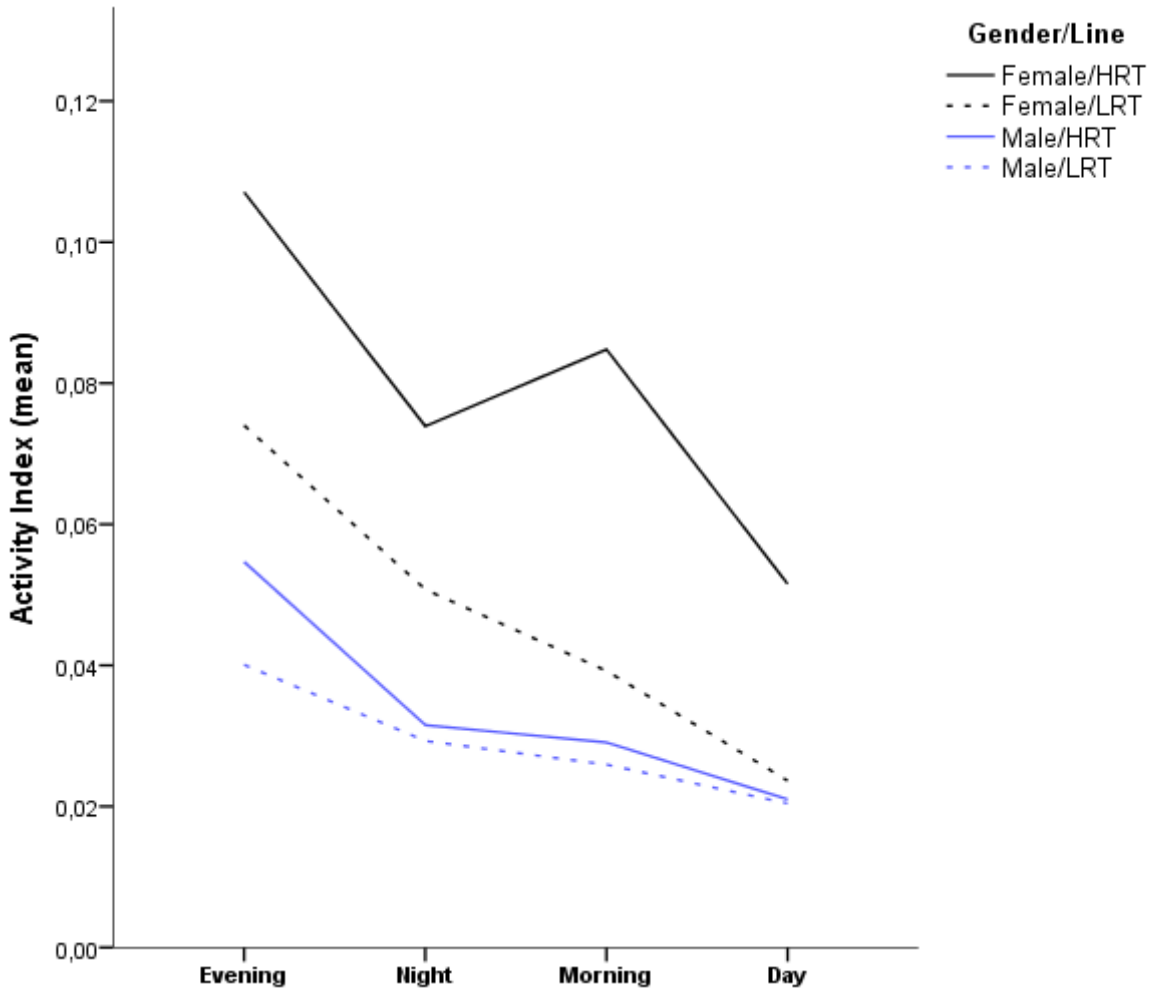


FIGURE 13. Spontaneuous activity by circadian rhythm. Rats were most active during evening and most inactive during day. (*** p<0.001)

To examine the variation of spontaneous activity during day among certain sex + line group, a variance variable was calculated. The mean variance is presented in table 2. Spontaneous activity had more variance in the group of female HRT rats.

Table 2. Variance in the spontaneuous activity was statistically higher in the group of female HRT rats (*p<0.05).

Sex	**Female**		**Male**	
Line	HRT	LRT	HRT	LRT
Group	0.0039*	0.0009	0.0004	0.0001



7.1.3 Sex differences

There was a highly significant difference between the genders in the average daily spontaneous activity (Independent-Samples Mann-Whitney test, $p<0.001$, N=70) (figure 14). The mean of average spontaneous activity in female rats was 0,066 (N=35) and 0,032 in male rats (N=35).

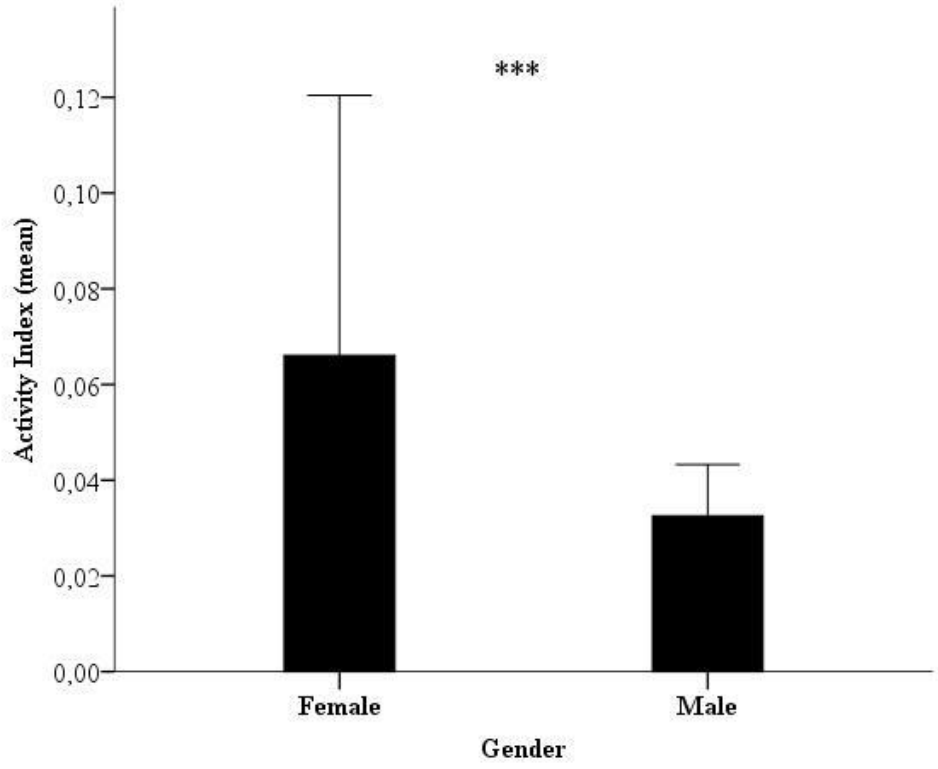


FIGURE 14. The spontaneous activity in female and male rats. The female rats were more spontaneously active than the male rats. (*** $p<0.001$)

The difference in the spontaneous activity between genders was highly significant in the three of the measuring periods (evening, night and morning), and significant during the day, the females being more active than males (figure 15).

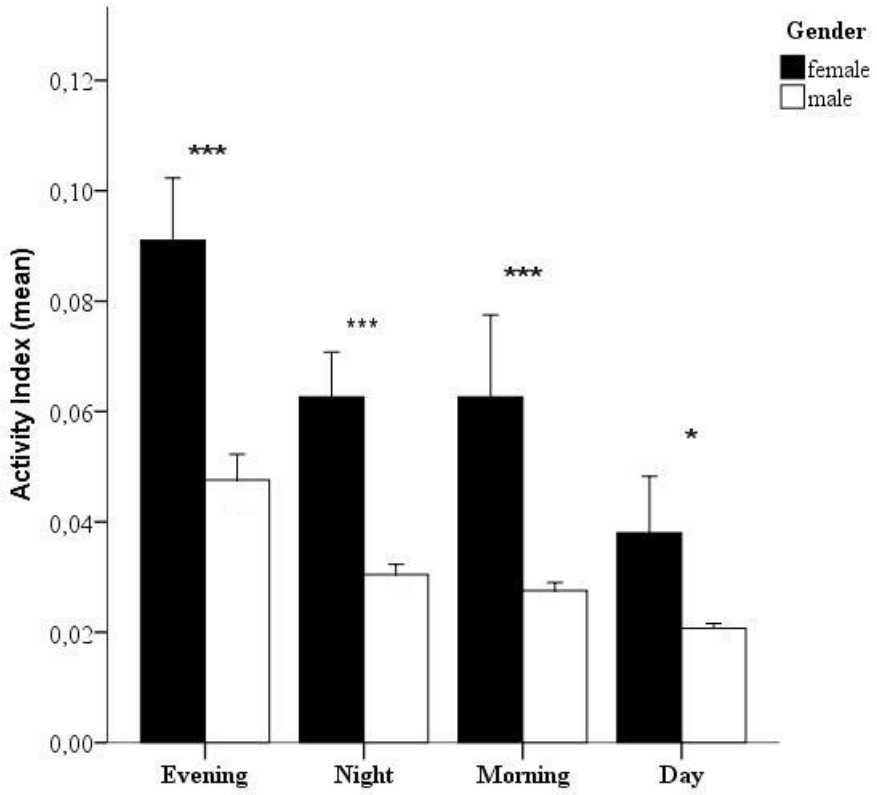


FIGURE 15. Spontaneous activity during different times of day between genders. Females are more active during evening, night and morning and statistically more active during day. (*** $p<0.001$, * $p<0.05$)

7.1.4 Between lines

HRT rats were more active than LRT rats. The mean activity index was 0.058 ±0.009 in HRT group (N=36) and 0.040 ±0.003 in LRT group (N=34). However, there were no significant differences in the average daily spontaneous activity between HRT and LRT. When spontaneous activity of the lines was compared according to the time of day, a statistically significant difference was found in one of the measuring periods: during morning ($p<0.05$,

N=70), the HRT rats being more active. Analyses were also run separately to both sexes but no statistically significant differences were found (figure 16).

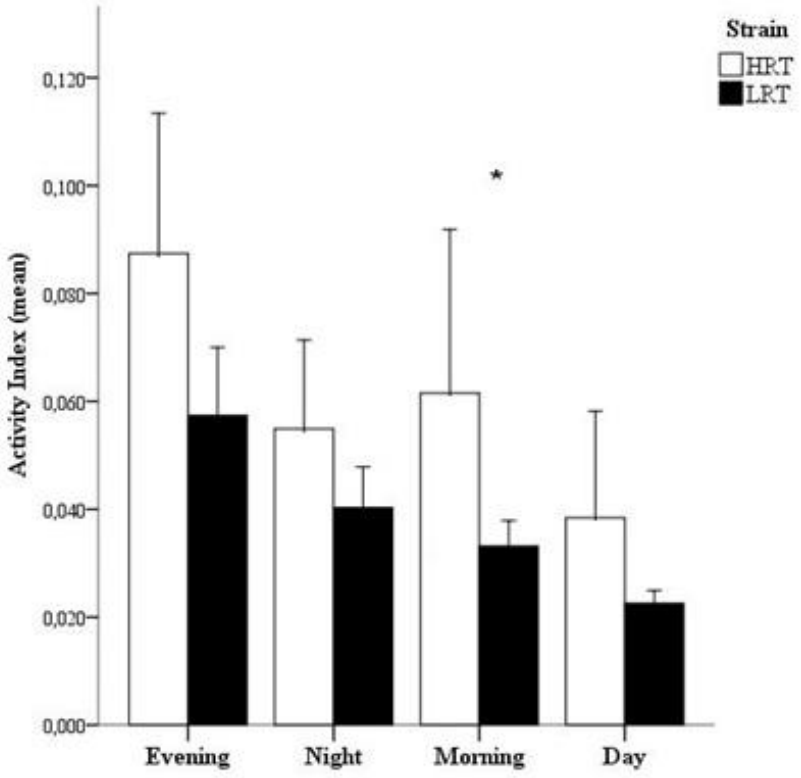


FIGURE 16. Spontaneous activity between rat lines (HRT/LRT). Statistically significant difference was only seen in the morning activity. (* $p<0.05$)

7.1.5 Between group differences

The rats in running wheel group were more spontaneously active than rats in the sedentary group. The mean spontaneous activity in RW group was 0.061 ± 0.001 and 0.033 ± 0.001 in SED, but this difference was not statistically significant. Though, a highly significant difference in spontaneous activity was found between the groups in female rats ($p<0.001$, N=35). No statistical difference was found in male rats between groups. Results are presented in figure 17.

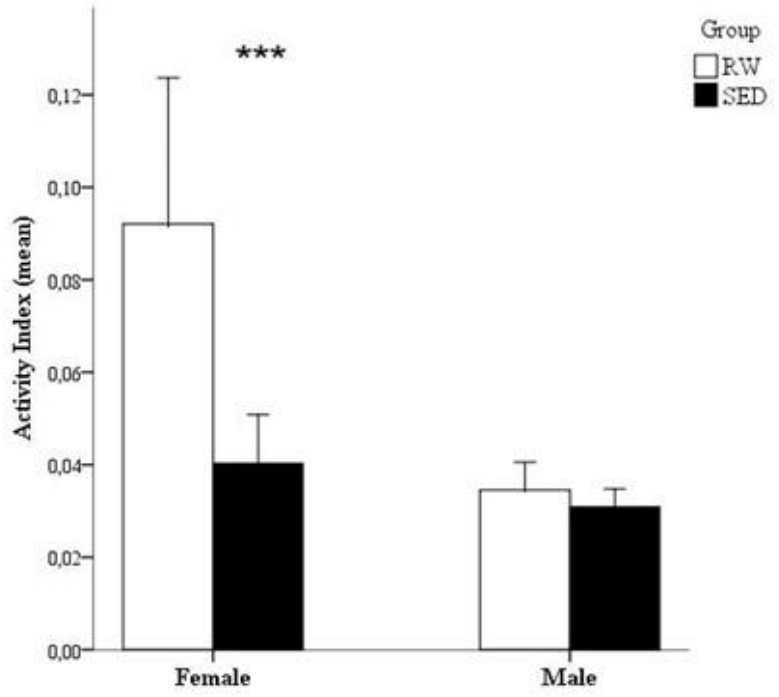


Figure 17. Spontaneous activity between groups in female and male rats. There was a highly significant difference between RW and SED female rats (*** $p<0.001$). No statistical difference in male rats.

Activity data according all groups and variables are presented in figure 18.

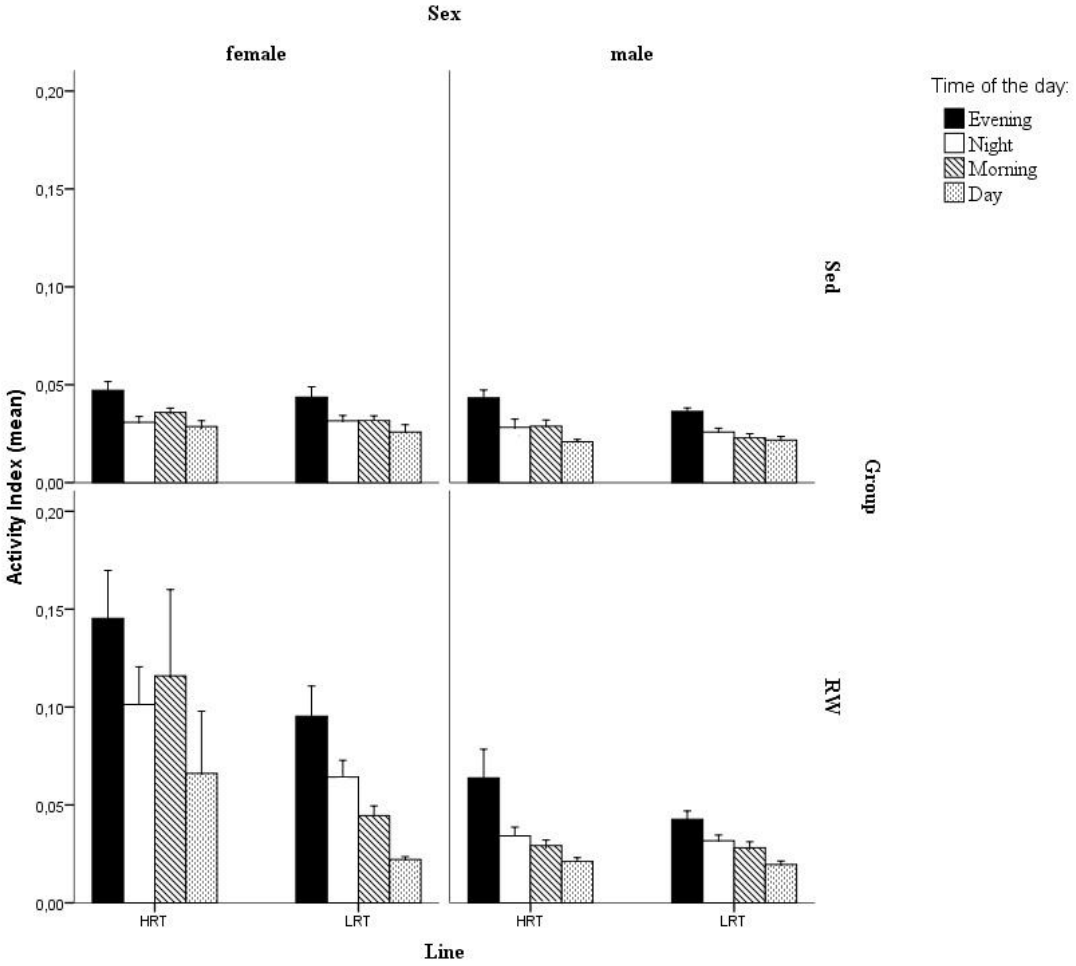


FIGURE 18. Spontaneuous activity in different lines stratified by group and sex

7.2 Adult Hippocampal neurogenesis

The number of new brain cells in hippocampus ranged from 96 to 1608, the mean number being 5489 ± 327. In HRT rats the mean was 492 ± 363 and in LRT rats 508 ± 287. Neurogenesis was greater in female HRT rats in the running group compared to the ones in sedentary group. Interestingly in the other groups (female LTR, male HRT and male LRT) neurogenesis was lesser in the running wheel groups. Statistical difference was found in the amount of neurogenesis between the rats in the running wheel group and in the sedentary group in case of female LRT rats ($p<0.05$). No statistically significant difference was found between the study groups in male rats or in case of HRT rats nor between sexes or lines.

Neither was there any correlation between the average daily spontaneous activity and adult hippocampal neurogenesis (Spearman's rho). The statistical tests were run separately to the two rat lines (HRT vs. LRT) and to rats in different study groups (RW vs. SED) and between sexes. The results of the neurogenesis are presented in table 5.

TABLE 4. Neurogenesis according to line, sex and study group. HRT female rats were the only group which benefit from running, in other groups neurogenesis was less in the running wheel group. A statistically significant difference was only found in female LRT rats between running wheel and sedentary group ($p < 0.05$)

Sex	Female				Male			
Line	HRT		LRT		HRT		LRT	
Group	SED	RW	SED	RW	SED	RW	SED	RW
New brain cells	531±546	645±427	572±213*	314±222*	435±204	384±164	627±370	469±271

Since the variance of activity in different times of day and hippocampal neurogenesis was higher in HRT female rats, the correlation between these variables was tested. No correlation between the variance of spontaneous activity with adult hippocampal neurogenesis was found. Correlation was tested between sexes, lines and groups. Especially the possible correlation in the group of female HRT rats was in interest, since this group was the only one which had greater neurogenesis due to running. Figure 19 visualizes these variables in sex+line groups.

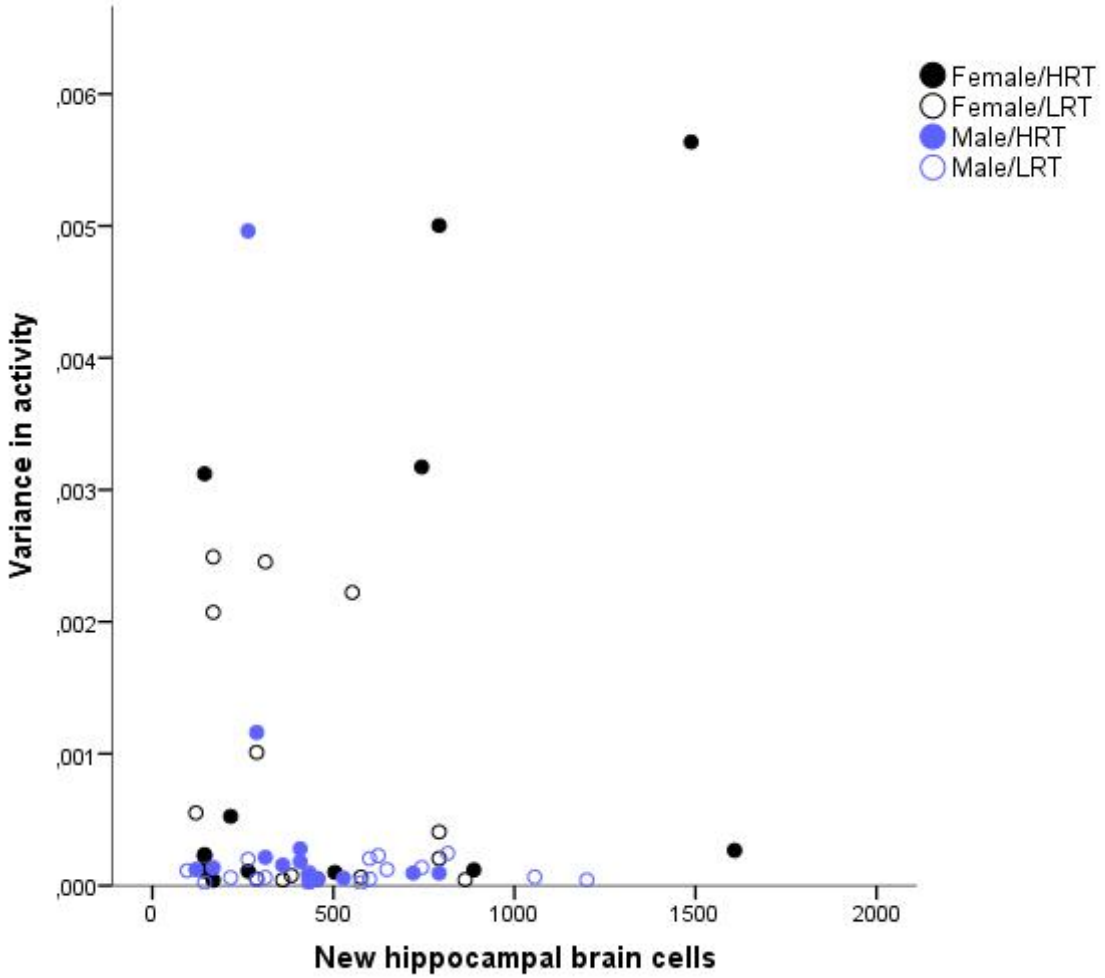


Figure 19. Variance in spontaneous activity and amount of new hippocampal brain cells presented in scatter plot. Grouping done according to sex and line. No correlation was found between the variables.

8 DISCUSSION

In this study the aim was to examine the spontaneous activity of HRT and LRT rats. To author's knowledge this has not been studied before. Another point of interest was to examine the effects of exercise on brain when exercising is started on older age after a long period of being sedentary and if the genetic factors affect the outcome that physical exercising generates. The studied rats, aged 1.6 years (represent human age of 45 years), represented two lines with differing genetic responsiveness to endurance training done at young age (Koch & Britton, 2001).

The results concerning spontaneous activity, support the hypothesis partly. It was observed that HRT rats were more active than LRT rats and that RW rats were more active than SED rats, but the differences in both cases were not statistically significant. A statistical difference was measured between sexes, the females being more active than male rats. In contrast to the hypothesis, voluntary exercise did not have positive effects on brain when exercising was started on older age. Also, neurogenesis was not more present in HRT rats compared to LRT rats.

Spontaneous activity. A positive correlation between spontaneous activity and wheel running was found. This is no surprise, since it is quite clear that when there is more running wheel activity the whole activity increases and vice versa. When correlation was run separately to both sexes, a statistically significant correlation was only observed in the group of female rats. This correlation might be because in there was an outlier with markedly high spontaneous activity. The activity data of this specific rat was checked in case of error in the data, but this was not observed. Other thing that must be considered is that the data from wheel running is collected daily during the study and an average daily amount of running is calculated from this data. It would be better to use the running wheel data and spontaneuous activity data from the exact same days, since spontaneous activity can be altered by many factors. Then the result would tell better about the correlation between the variables. Mean values are not the best ones to use in case of examining correlations, because there might be significant differences in the variables.

The rats were statistically highly more active during the darker periods of the day (night, evening and morning). The rats were most inactive during the bright day. Main reason for these differences lies in the fact that rats are nocturnal animals by nature (Whisshaw et al. 2005). These findings support previous knowledge (Suckow et al 2005; Whisshaw et al. 2005). Circadian rhythm is controlled by the master clock, and many factors affect its functioning. One factor that might be relevant in this study is the high age of the rats. According to Richter (1922) the differences in activity damper with age and with younger rats the differences might be more radical. The rats were about as active during evening and morning – around the time when lights were turned on or off. This kind of trend has also been seen by Suckow et al (2005). Also in this study the female rats were statistically highly more active than male rats like it's been known before (Whisshaw et al. 2005).

HRT rats were more active than LRT rats, but the difference was not statistically significant. When the activity of the rat lines was compared according to the time of the day, a statistically significant difference was found in the morning activity. The spontaneous activity of HRT and LRT rats has not been studied before. According to these results it seems that there is no difference in the activeness between these lines. One thing that might affect the results is that the rats had been sedentary for a long time. The difference between the lines could appear more strongly if the rats had been active earlier in their life.

The rats in the running wheel group were more spontaneously active compared to rats in sedentary group, but these differences were not statistically significant. When the spontaneous activity was examined between groups of subjects representing the same sex, the result show that female rats in running wheel group were statistically highly more active than female rats in sedentary group. According to a previous study by Karvinen et al. (2015) and Novak et al. (2012), rats with running wheels should be more active than rats in sedentary group. Hence, the results of this study support these findings only partly. One reason the difference between the lines in this study was not so evident, might be in the old age of the rats. In the study of Karvinen and colleagues (2015) the subject rats were around nine months old, whereas in this study the rats were almost twice as old.

One of the key findings in spontaneuous activity was in the variation of activity in HRT female rats. This group stood out from the other groups, since HRT female rats had more fluctuation in their daily activity pattern. So, HRT female rats were not only the most active ones when spontaneous activity was measured altogether, but they also had most rhythmic trend in the activity. They were most active during evening but another burst in activity was seen in the morning measurement point. Same kind of trend was seen in the group of HRT male rats, but in a clearly milder manner. The activity of LRT rats was plateaued in a way that they were most active during the evening and their activity followed to decrease until the next evening when it increased again. The HRT female rats had more distinct circadian rhythm in their running trend. In this study only old rats were observed. It would be interesting to study these trends between young and old rats and examine if HRT rats are able to retain the circadian activity rhythm longer than LRT rats or do these lines have distinctive differences throughout life. And whether these lines have differences in the circadian rhythm factors and proteins. Studies with rodents have shown that aging has effects on the circadian rhythm (Morin, 1988; Scarbrough, 1997; Valentinuzzi et al. 1997).

Other factors that might affect the result are possible stress (Gorka et al. 1996; Harper et al. 1996) caused by the study protocol and tests done to the subjects. Rats are social animals by nature and they are sensitive to social isolation (Hall, 1998). Isolation may affect the spontaneuous activity (Koolhaas, 2010). In adulthood, long term social isolation can induce behavioural disturbances such as increased activity, anxiety or depression (Arakawa, 2005: Malkesman et al. 2006; Silva et al. 2003). Since rats in this study were kept in single-housed cages, it is possible that it affected the rats' behaviour. All of the rats were housed in a same way, so the possible effects should be seen in each rat.

The shipment of the rats from United States to Jyväskylä in this study probably did not affect the circadian rhythm of the rats. It usually takes 3 to 4 weeks for the circadian rhythm to re-entrain and in this study, there was over two months between the shipment and the study (Whisshaw & Kolb, 2005).

One thing also to take into consideration is that rat's single normalized body mass value was used to calculate spontaneous activity. Changes in the weight might have affected the activity index. The effect is dependent on the greatness in the body mass changes during the measurement period.

Adult hippocampal neurogenesis. Against hypothesis, no correlation was found between spontaneous activity and adult hippocampal neurogenesis. A statistical difference in AHN was only seen in between sedentary and running wheel rats in female LRT rats. No statistical differences were found in other groups (not between genders, lines or sedentary and running wheel). Interestingly, the mean of neurogenesis was higher (not statistically significant) in the sedentary group compared to wheel running group both in female and male rats. Also, the standard deviation in every group was high. This was against the hypothesis that HRT rats would have more neurogenesis, like in previous the study by Nokia et al. (2016). Though, results of this study are in parallel with the results of the Master's thesis by Harri and Heiskanen (2014) who discovered that hippocampal neurogenesis is not dependent on rat line.

When studied with young mice (10 weeks old) the mice with running wheels had significantly higher number of newborn neurons than mice in the control group (Lee et al. 2013). The differing result in this study can be explained by the age difference of the rodents. In a study by van Praag et al. (2005) the result was that when mice started wheel running in old age (1.58 years), the number of brain cells was higher compared to sedentary mice. Perhaps the contrary findings can be explained by the difference between mice and rats. The rats used in our study were a bit older (1,6 years). Though the age difference is so little, in the age scale of rats it might be pivotal. It has been studied that cell proliferation and cell survival in rodents decline sharply with age (Kuhn et al. 1996; Rao et al. 2006). According to study made by McDonald et al. (2005) neurogenesis can decrease even by 94% in rodents between the time from their adolescence to middle age. This slight age difference might give some explanation.

Because the variables were not normally distributed non-parametric test were used. The non-normal distribution and skewness were probably due to the aging affects and the fact that the speed of aging varied in the different groups. Non-parametric tests are not as sensitive to flag

out significances as parametric tests, and it is possible that this affected the results. Because the skewness of the data was so remarkable, it was not possible to convert the variables to be normally distributed.

Interestingly, both hippocampal neurogenesis and daily variation in spontaneous activity was higher in HRT female rats, but no correlation was found between these variables. HRT rats might have genetical factors which enhance their neurogenesis especially with activity. Some of these factors may be linked to genetic circadian regulation. The link between these positive outcomes which were seen in HRT female rats and circadian rhythmicity should be examined more profoundly though correlation was not evident in this study. Link between circadian rhythmicity and brain health have been seen in previous studies (Bouchard-Cannon et al. 2013; Holmes et al. 2004).

According to the result of this study, it seems that there is no benefit to start physically active lifestyle at older age. Actually, the results support sedentary lifestyle, since neurogenesis was more present in sedentary female rats compared running wheel group. If the age of the rats' are compared to human age, this would mean that physical activity started after the age of 45 does not increase neurogenesis.

It has to be taken into consideration that the brain mechanisms may not be the same in humans and in rats or other rodents. Also, the benefits of exercise are tested in laboratory environment and there is no evidence that the benefits will extend outside the laboratory to everyday cognitive functioning. (Kramer et al. 2007.) The effects of exercise are not independent in a way that all factors may influence the outcomes together. In fact, it might be that the positive effects that have been noted in human studies in the field of exercising and brain health, are due to other factors than exercising. One possibility is that the social aspect of exercising brings up its benefits.

The effects of exercising to brain health are fascinating and surely need more studying. When genes are added to the equation even more research is needed. In a world with aging population, the role of brain health comes more important with the factors influencing it.

Another interesting topic for future research is to examine whether the difference in genetic responsiveness appears when exercising is started in older age.

9 CONCLUSIONS

The aim of this study was to examine the role of spontaneous activity on brain health when the activity is started in older age. The rats represented two lines (HRT and LRT) which were selectively bred to have difference in their inherent responsiveness to physical exercise done at young age. The objective was to examine the spontaneous activity of HRT and LRT rats and its effects on adult hippocampal neurogenesis and possible differences in neurogenesis between these two lines. The spontaneuous activity between these two lines, was not been studied before.

The spontaneous activity was measured by using a ground reaction force plate method. The gathered activity data was sampled and activity indexes were calculated to hourly means and further to mean values in different periods of the day. The analysis periods were morning (6 am -10 am), day (11 am- 5 pm), evening (6 pm-10 pm) and night (11 pm – 5 am). For the neurogenesis analyses, the rats' brains were collected and the hemispheres and cerebellum separated. After laboratory operations and Cresyl Violet staining new brain cells in the granular cell layer and in the hilus on dentate gyrus were calculated.

The results support earlier studies of female rats being more active than male rats and that rats overall are more active during dark periods. HRT rats were measured to be more active than LRT rats, but these results were not statistically significant. HRT rats had more variation in daily activity when measurement periods were examined. Especially HRT female rats had fluctuations in the activity. HRT female rats also were the only group which benefit from running when neurogenesis was measured. Although no correlation was seen between the variation of activity and neurogenesis. The result of this study indicate that physical exercise started in old age does not increase neurogenesis and that the inherent component of responsiveness to physical activity does not impact the amount of neurogenesis in old age.

Although, these findings suggest that physical activity started in old age is not beneficial in case of neurogenesis, physical activity may have other physiological benefits. Only one relation of physical exercise started in old age was examined in this study. Exercising is

beneficial also for respiratory and circulatory system and gives social experiences and nourishes mental health. Also, result of rat studies should not be reflected to humans straightforwardly.

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