INFLUENCE OF VOLUNTARY WHEEL RUNNING ON SEIZURE MODULATION AND GALANIN GENE EXPRESSION IN THE RAT LOCUS COERULEUS

by

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(Under the Direction of Philip V. Holmes)

ABSTRACT

Voluntary wheel running is associated with a reduction in seizure severity, an effect that in part arises from the activity of galanin, an inhibitory neuropeptide whose gene expression in the locus coeruleus is sensitive to exercise. The effect of three weeks of free access to activity wheels on the severity of kainic acid-induced seizures was investigated. Associated measures of seizure severity, a modified Racine severity scale and frequency of spike-wave discharges with electroencephalography, were used to characterize seizure severity at a i.p. dose of 10mg/kg of kainic acid. The difference in spike-wave frequency approached significance between exercising and sedentary rats. To investigate the influence that aerobic capacity may have on any exercise-induced upregulation of galanin in the locus coeruleus, rats selectively bred to demonstrate greater or lesser aerobic capacity, and Sprague-Dawley rats, were provided with free access to activity wheels for three weeks. Galanin and tyrosine hydroxylase gene expression in the locus coeruleus was quantified using in situ hybridization and optical density analysis. The different strains of rats did run differently over the study, and both strain and exercise condition did affect galanin mRNA expression, but did not influence tyrosine hydroxylase gene expression. No strain differences in galanin or tyrosine hydroxylase expression was observed in sedentary rats. Galanin expression and overall running distance were significantly correlated. This data

demonstrates that exercise-induced galanin upregulation is related only to the amount of running, and that the capacity to run does not have any additional influence.

INDEX WORDS: Seizure, galanin, EEG, kainic acid, activity wheel, locus coeruleus

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DEDICATION

This work is dedicated with love to my parents, Heather, Anna, Jason, and Sheila.

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I wish to thank my dissertation committee members for their advice, support, and patience. Most of all I would like to express a profound thanks to my advisor Philip Holmes, who truly demonstrates excellence in teaching and mentorship.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

GENERAL INTRODUCTION

Exercise produces many benefits with regard to mental health, benefits which are underscored by an associated change in the nature of signaling in the brain. It is linked with a reduction in self-reported depression severity (Dunn, Trivedi, Kampert, Clark, & Chambliss, 2005), and various rodent models of depression support this effect (Chambliss, Van Hoomissen, Holmes, Bunnell, & Dishman, 2004; Dishman et al., 1997). In addition to these effects, physical activity can also enhance processes associated with learning (Anderson et al., 2000; Farmer et al., 2004) and can augment measures of neuroprotection from conditions involving cell death. Exercise reduces severity of stroke (Stummer, Weber, Tranmer, Baethmann, & Kempski, 1994), Alzheimer's disease (Nichol, Parachikova, & Cotman, 2007), and Parkinson's disease (Mabandla, Kellaway, St. Clair Gibson, & Russell, 2004), and similarly promotes neuroprotective processes in seizure (Reiss, Dishman, Boyd, Robinson, & Holmes, 2009).

Many of the beneficial effects of exercise have been linked to discrete changes in neurotransmitter systems, and norepinephrine (NE) is highly implicated in many of these effects. However, the type of exercise in animal models influences the interpretation of any observed effects on neurotransmitter systems. For example, NE production in the locus coeruleus (LC) is largely unaffected by exercise, unless the mode of exercise is stressful. Forced running on a treadmill provides similar exercise effects as voluntary running (Holmes, Yoo, & Dishman, 2006; O'Neal, Van Hoomissen, Holmes, & Dishman, 2001), integrated with an additional effect of stress (Timofeeva, Huang, & Richard, 2003; Yanagita, Amemiya, Suzuki, & Kita, 2007). NE

activity may be affected by treadmill running (Tümer et al., 2001), but voluntary access to a cage wheel does not influence tyrosine hydroxylase (TH) expression in the LC (Soares et al., 1999). It does however affect the expression of the inhibitory neuropeptide galanin (GAL) in the LC (Holmes, et al., 2006; Van Hoomissen, Holmes, Zellner, Poudevigne, & Dishman, 2004), whose activity is associated with some of the positive effects of exercise.

Neuropeptides are widely colocalized with classical neurotransmitters. GAL is colocalized with NE in the LC (Hökfelt et al., 1987; Lundberg & Hokfelt, 1983) and is released under conditions of higher rates of LC activity (Consolo et al., 1994). By activation of potassium channels (Dunne, Bullett, Li, Wollheim, & Petersen, 1989; Pieribone et al., 1995) and adenylyl cyclase inhibition (He et al., 2005), GAL supports a decrease in membrane potential and neuronal activity, with an associated reduction in hyperexcitability (Mazarati & Lu, 2005). GAL exerts these effects via projections from the LC to the hippocampus (Ungerstedt, 1971), which is especially sensitive to conditions of hyperexcitability(Santhakumar, Ratzliff, Jeng, Toth, & Soltesz, 2001). By action via the different GAL receptor subtypes, the neuropeptide can reduce the severity of seizures and increase the latency to onset of seizure following seizure induction by, for example, electrical stimulation (Mazarati & Lu, 2005). Considering these neuroprotective and anticonvulsive qualities of GAL, it is of interest to characterize the difference in seizure quality between exercising and sedentary rats.

The change in GAL expression that accompanies exercise is related to the amount of running; that is, more running generally means more GAL expression in the LC (Eisenstein & Holmes, 2007). With the development of models of different aerobic capacities, tools are available by which to further characterize the relationship between running and GAL expression (Koch & Britton, 2001), as ability to run may be involved in GAL expression. Rats selected for

greater (high-capacity runners, or HCR) or lesser aerobic capacity (low-capacity runners, or LCR) and bred over generations demonstrate dramatic differences in running (Koch & Britton, 2008). This difference may be related to a selection-mediated modulation of how the rats transport and utilize oxygen in skeletal muscle (Gonzalez et al., 2006; Howlett et al., 2002; Howlett et al., 2009).

The present studies seek to further characterize how running contributes to the neuroprotective effects of exercise. In the first study (Chapter 3), rats with free access to running wheels for three weeks were then implanted with subdural electroencephalography probes. They received systemic kainic acid injections, a potent kainite receptor agonist, and the resultant seizure severity was determined by behavioral rating and EEG analysis. The second study (Chapter 4) employed HCR and LCR rats provided free access to activity wheels for three weeks. Following the exercise protocol, the GAL and TH activity in the LC was determined with in situ hybridization. Running behavior of these rats was also examined.

CHAPTER 2

LITERATURE REVIEW

Exercise

Exercise influences many aspects of brain function, and produces beneficial neurophysiological changes. Indeed exercise appears to produce benefits with regard to stress responses (Salmon, 2001), and it reduces depression severity in a manner dependent upon energy expenditure (Dunn, et al., 2005). These effects on depression and stress have also been verified using animal models; in foot shock studies of both rats and mice with free access to running wheels, exercising animals demonstrated decreased escape latencies compared to sedentary animals (Dishman, et al., 1997; Duman, Schlesinger, Russell, & Duman, 2008; Greenwood et al., 2003), an effect similar in magnitude to that observed with antidepressant administration (Duman, et al., 2008). Activity wheel running also improved Sprague-Dawley male copulatory performance in a clomipramine model of depression (Yoo, Tackett, Crabbe, Bunnell, & Dishman, 2000) and reduced ejaculation latency in imipramine-treated, olfactory-bulbectomized rats (Chambliss, et al., 2004).

Exercise produces many protective effects under a variety of circumstances with regard to brain function deficits revolving around neuronal cell death. Treadmill running has been demonstrated to protect against damage in a focal stroke model; investigators found a reduced infact volume following occlusion of the middle cerebral artery after 3 weeks of running (Ding et al., 2004), although another study did not identify a substantial reduction in infact volume until

after 12 weeks of running (Ang, Wong, Moochhala, & Ng, 2003). Treadmill running also reduced spontaneous recurrent seizures in rats following pilocarpine administration (a model of epilepsy) (Arida, Scorza, Ferreira Dos Santos, Peres, & Cavalheiro, 1999), and modulated CA1 hyperresponsiveness in the epileptic rats (Arida et al., 2004).

Compared to sedentary rats, long-term chronic treadmill running does not produce the same behavioral effects on the elevated plus maze and open field as voluntary wheel running however (Burghardt, Fulk, Hand, & Wilson, 2004), indicating that the two modes of exercise have divergent effects. Furthermore, it is important to mention that compared to spontaneous running, forced running produces stress effects in addition to and confounded with any exercise benefits (Timofeeva, et al., 2003; Yanagita, et al., 2007). Voluntary access to an activity wheel then would bear more comparable utility in an examination of exercise effects per se, since the running distance is roughly equivalent anyway (Yanagita, et al., 2007).

Activity wheel running is associated with a reduction in the severity of seizures in more than one animal model of temporal lobe epilepsy; three weeks of voluntary wheel running reduced kainic acid seizure severity at an intracerebroventricular dose of 0.2 μg (Reiss, et al., 2009). Kainic acid preferentially lesions specific hippocampal areas (CA1, CA3) in a characteristic manner (Nadler, Perry, & Cotman, 1978; Schwob, Fuller, Price, & Olney, 1980), not unlike pilocarpine administration (Mello et al., 1993). Voluntary wheel running reduces recurrent seizures from pilocarpine injection, even if the running occurs after the first initiation of status epilepticus with the injection (Arida, Scorza, Terra, Cysneiros, & Cavalheiro, 2009).

The neuroprotective effects of wheel running is not isolated to just models of epilepsy; Long-Evans rats with free access to cage wheels prior to and following 6-hydroxydopamine injection, which produces parkinsonian symptoms (Ungerstedt, Avemo, Avemo, Ljungberg, &

Ranje, 1973), showed a sparing of striatal dopaminergic neurons as measured by a lower apomorphine-induced rotation frequency (Mabandla, et al., 2004). In a mouse model of spinal cord injury, three weeks of voluntary wheel running prior to contusion injury to T9 led to improved locomotor outcome measures (Engesser-Cesar, Anderson, Basso, Edgerton, & Cotman, 2005). Just two weeks of wheel running in Mongolian gerbils led to a dramatic reduction in deaths of running gerbils in the days following single 15- or 20-minute bouts of forebrain ischemia (Stummer, et al., 1994). Access to running wheel has also led to improvements in radial arm water maze performance in a transgenic mouse model of Alzheimer's disease (Nichol, et al., 2007).

Activity wheel running is associated with an enhancement of learning processes and the impact of aging on these processes. Rats with access to activity wheels reached criterion performance on the eight-arm radial maze with substantially (30%) fewer trials than sedentary rats (Anderson, et al., 2000). Wheel running enhances neurogenesis and long term potentiation (LTP) in the dentate gyrus (DG) of Sprague-Dawley rats (Farmer, et al., 2004), and also improves performance on the Morris water maze task (van Praag, Christie, Sejnowski, & Gage, 1999). Even the normal deficits in Morris water maze performance associated with kainic acid are improved after voluntary wheel running (Gobbo & O'Mara, 2005). In older mice (19 months), 45 days of voluntary wheel running enhanced performance on the Morris water maze test compared with age-matched sedentary mice (van Praag, Shubert, Zhao, & Gage, 2005). Voluntary running also increased retention on the passive avoidance task in various groups of older rats, grouped by age (and in order of increased latency on the task): 20-24 months, and 28-30 months, and 10-14 months (Samorajski et al., 1985).

Many of the effects of exercise manifest due to changes in the robustness of cellular survival and proliferation pathways, such as phosphoinositide 3-kinase and mitogen-activated protein kinase (Chen & Russo-Neustadt, 2005; Shen, Tong, Balazs, & Cotman, 2001). Voluntary running increases expression of vesicular proteins synapsin and synaptophysin (Vaynman, Ying, & Gomez-Pinilla, 2004; Vaynman, Ying, Yin, & Gomez-Pinilla, 2006), and is strongly associated with alterations in the activity of trophic factors such as brain-derived neurotrophic factor (BDNF) (Farmer, et al., 2004; Van Hoomissen, Chambliss, Holmes, & Dishman, 2003), an enhancement that appears to be age-dependent (Adlard, Perreau, & Cotman, 2005).

Norepinephrine and the Nucleus Locus Coeruleus

NE synthesis begins with the amino acid tyrosine, which is hydroxylated to 3,4-dihydroxyphenylalanine (L-DOPA) in a reaction catalyzed by TH (Nagatsu, Levitt, & Udenfriend, 1964), the rate-limiting step in NE synthesis (Levitt, Spector, Sjoerdsma, & Udenfriend, 1965). In a decarboxylation reaction, L-DOPA is converted by DOPA decarboxylase to 3,4-dihydroxyphenylethylamine (dopamine) (Holtz, 1959), which is then converted to NE by dopamine-beta-hydroxylase (Kaufmann & Friedman, 1965) and electron-donating ascorbic acid (Menniti, Knoth, & Diliberto Jr, 1986). NE binds alpha and beta adrenergic receptors (Minneman, Pittman, & Molinoff, 1981), though more strongly with alpha adrenergic receptors compared to epinephrine (Ahlquist, 1948). Alpha1-adrenergic receptors are postsynaptic, while alpha2-adrenergic receptors are presynaptic (Berthelsen & Pettinger, 1977; Langer, 1974). Beta adrenergic receptors activate adenylyl cyclase, alpha1-adrenergic receptors activates phospholipase C, and alpha2 inhibit adenylyl cyclase and activate phospholipase C (for review see Kobilka, 1992).

The principle noradrenergic neurons in the nervous system are found in the LC and certain medullary nuclei (A1, A2, A5, and A7) (Holmes & Crawley, 1995). ICV injection of radioactively labeled tyrosine resulted in an accumulation of radioactive NE in the LC, an amount that increased with time following injection (Kuhar, Roth, & Aghajanian, 1972). Located in the dorsal tegementum, the LC nuclei border on the fourth ventricle and pontine central gray medially, the superior cerebellar peduncle dorsolaterally, and the mesencephalic tract of the trigeminal nerve laterally (Amaral & Sinnamon, 1977; Russell, 1955).

There are five major projections of the LC: to the hypothalamus (via the dorsal noradrenergic bundle), the thalamus (via the central gray dorsal longitudinal fasciculus), and the cerebrum (via the ventral tegmental-medial forebrain bundle), as well as the cerebellar cortex and spinal cord (Holmes & Crawley, 1995). These projections are largely spatially organized in the LC as hippocampal (both dorsal and ventral) projections originate in the dorsal portion of the nucleus and its posterior pole, spinal cord projections originate in ventral-posterior aspects, and hypothalamic projections originate in the anterior pole (Loughlin, Foote, & Bloom, 1986). Cerebral and cerebellar fibers are less clustered, with cerebral fibers found in central aspects of the nucleus and cerebellar fibers found throughout the dorsal-ventral LC and prominently in the ventromedial portion (Loughlin, et al., 1986). Importantly, the LC comprises the sole noradrenergic innervation of the hippocampus (Morrison, Grzanna, Molliver, & Coyle, 1978; Pickel, Segal, & Bloom, 1974; Ungerstedt, 1971).

Afferent innervation of the LC arises largely from two nuclei in the rostral medulla, the paragigantocellularis (PGL) and ventromedial prepositus hypoglossi (PRH), with minor contributions from the paraventricular nucleus (PVN) in the hypothalamus and spinal intermediate gray (Aston-Jones, Ennis, Pieribone, Nickell, & Shipley, 1986). Additional

retrograde labeling has identified inputs from the preoptic area, dorsal to the supra optic nucleus, posterior hypothalamus, Kolliker-Fuse nucleus, and mesencephalic reticular formation (Luppi, Aston-Jones, Akaoka, Chouvet, & Jouvet, 1995). The extranuclear dendrites of the LC (Swanson, 1976) receive 5-HT, GABAergic, and catecholaminergic inputs (including somatdendritic NE release) that differentially adjust to various conditions such as stress, pain, opiod withdrawl, as well as fluctuations in blood pressure (Singewald & Philippu, 1998).

Reciprocal connections to rostral medulla nuclei, as well as hypothalamic nuclei, underlie the mechanism by which LC influences cardiovascular homestasis (Singewald & Philippu, 1996). Electrical stimulation of the LC causes an increase in blood pressure (Przuntek & Philippu, 1973), as well as enhanced NE release in the posterior hypothalamic nucleus (Philippu, Dietl, & Sinha, 1979). A reduction in blood pressure also causes an increase in NE release in areas such as frontal cortex and posterior hypothalamus (Smagin, Swiergiel, & Dunn, 1994); NE release in the LC is diminished under hypertensive conditions (Schneider, Singewald, & Philippu, 1995). These effects will not occur without a functional paragigantocellularis (Aston-Jones, Chiang, & Alexinsky, 1991).

LC GAL is also implicated in the regulation of cardiovascular homestasis, as GAL release in the nucleus tractus solitarius via projections retrogradely labeled from the LC participates in the suppression of baroreceptor reflex (e.g. suppression of heart rate under hypertensive conditions) (Shih, Chan, & Chan, 1996). GAL release in the PVN has the opposite effect, suppressing the PVN-induced baroreceptor reflex (Chen, Chan, & Chan, 1996).

Individual stressors such as foot shock (Cedarbaum & Aghajanian, 1978a), loud noise (Abercrombie & Jacobs, 1987), and tail pinch (Aston-Jones & Bloom, 1981b) strongly influence NE release in target areas such as cortex, hippocampus, and amygdala (Stanford, 1995). Stress

also elicits somatodendritic NE release in the LC, which modulates activity via the α_2 autoreceptor (Singewald & Philippu, 1998). This evidence suggests that the LC participates in integrating incoming information regarding stress and cardiovascular state to influence behavioral and cardiovascular response.

The LC is involved in the regulation of cortical arousal and attention in a complicated fashion (Aston-Jones, Rajkowski, & Cohen, 1999). Recordings of LC neurons were analyzed in conjunction with cortical EEG activity during sleep in rats (Aston-Jones & Bloom, 1981a): the neurons discharge in a predictable pattern during stages of sleep, with the highest rate of activity during wakefulness, a decline during slow wave sleep, and nearly no activity during REM sleep. LC neurons fire in conjunction with bursts of EEG activity during slow wave sleep, predictably decreasing in activity just prior to and following the EEG bursts (Aston-Jones & Bloom, 1981a). LC activity characteristically coincides with EEG indicators of wakefulness, but prior to EMG indications.

Behaviorally, LC cells show distinct patterns of phasic and tonic activity, each associated with focused and scanning attention respectively in a visual discrimination task (Aston-Jones, et al., 1999). Corticotropin-releasing hormone (CRH) release from hypothalamic PVN projections serves to influence specifically tonic LC activity (Valentino & Foote, 1988), implicating a shift to scanning attention under conditions that promote CRH release. Glutamate-induced phasic activation of the LC produced changes in EEG recordings from the DG, exhibiting transient increases in theta power and reduced beta-gamma frequencies, as well as promotion of long-term potentiation (Brown, Walling, Milway, & Harley, 2005), implicating a memory process. The LC also releases more NE with each spike when phasically activated (Florin-Lechner, Druhan, Aston-Jones, & Valentino, 1996). It has been proposed that LC activity underlies attentional or

cognitive shifts, such as are involved in changes to stimulus-reinforcement contingencies or an unexpected event (Bouret & Sara, 2005).

Interestingly, rats will engage in self-stimulation with an electrode impanted in the LC, an effect abolished with a NE synthesis inhibitor (Ritter & Stein, 1973). Low frequency electrical stimulation of the LC elicits a long latency, lasting inhibition of hippocampal cell activity (Amaral & Sinnamon, 1977; Segal & Bloom, 1974). A similar hippocampal inhibition is also observed in the presence of an intense auditory stimulus, an effect potentiated by LC stimulation (Segal & Bloom, 1976). However, the LC acts in a fashion more complicated than simply to inhibit; in cortical neurons, NE enhances excitatory responses to ACh as well as inhibitory responses to GABA while also reducing background discharge, in effect increasing the "signal-to-noise-ratio" (Servan-Schreiber, Printz, & Cohen, 1990; Waterhouse, Moises, & Woodward, 1980). This phenomenon occurs in several areas that receive LC fibers, such as hippocampus, midbrain, thalamus, and spinal cord (Aston-Jones, et al., 1991; Aston-Jones, et al., 1999).

The Neuropeptide Galanin

Neurotransmitters such as NE or acetylcholine are often colocalized in neurons with other neurotransmitters or peptides (Hökfelt, et al., 1987; Lundberg & Hokfelt, 1983). For instance, neuropeptide Y and GAL both exist with NE in the cells of the LC (Holmes & Crawley, 1995, 1996; Holmes, de Bartolomeis, Koprivica, & Crawley, 1995; Melander et al., 1986a). GAL immunoreactive neurons are found mainly in central and dorsal LC, while NPY immunoreactive neurons are found mainly in the dorsal LC (Holets, Hokfelt, Rokaeus, Terenius, & Goldstein, 1988). Small cells surrounding the LC as well as A4 cells also demonstrate GAL-like immunoreactivity (Melander, et al., 1986a). GAL, as with other neuropeptides, is released under

conditions of higher activity than normal, in a calcium-dependent manner (Consolo, et al., 1994; Iversen, Lee, Gilbert, Hunt, & Emson, 1980; Lundberg & Hokfelt, 1983).

GAL receptors are G protein-coupled receptors linked to inhibition of adenylyl cyclase (He, et al., 2005). All three GAL receptor subtypes inhibit adenylyl cyclase (Habert-Ortoli, Amiranoff, Loquet, Laburthe, & Mayaux, 1994; Hobson et al., 2008; Smith et al., 1998). GALR1 mRNA is found in the lateral part of the central gray at the level of the pons, and cells in the dorsal parabrachial nucleus; GALR2 mRNA is strongly observed in the granule cells of the DG (Xu, Shi, & Hokfelt, 1998). These findings were further verified with an extensive characterization of GALR1 and GALR2 mRNA in the rat CNS, showing GALR1 mRNA most prominently in the olfactory bulb and nucleus of the accessory olfactory tract, throughout the septum and diagonal band, CA1 and subilculum in the hippocampus, throughout the thalamus and hypothalamus, and also in the LC and large cells of the dorsal root ganglia; GALR2 was identified most strongly in the DG of the hippocampus, mammillary nuclei, and small cells of the dorsal root ganglia, among other areas including the olfactory system and hypothalamic nuclei (O'Donnell, Ahmad, Wahlestedt, & Walker, 1999). The highest levels of GALR3 mRNA have been found in the hypothalamus, pituitary, and medulla of the rat (Smith, et al., 1998), and also the dorsal paragigantocellular field (Mennicken, Hoffert, Pelletier, Ahmad, & O'Donnell, 2002).

GALR1-knockout mice demonstrate an impairment in trace fear conditioning (Wrenn et al., 2004), an effect similar to that observed in mice that overexpress GAL (Kinney et al., 2002); these seemingly contradictory effects may underlie a self-regulation of GAL activity, thus GALR1 may serve as inhibitory autoreceptors. This has been supported electrophysiologically: GAL applied to LC neurons produced hyperpolarization followed by a decrease in membrane

resistance, facilitated by an increase in potassium conductance (Pieribone, et al., 1995). GAL signaling directly modulates GALR1 in the LC, as the GALR1 will increase proportionally to any increase in adenylyl cyclase activity; but the same effect is not observed with GALR2 or GALR3 (Hawes, Brunzell, Wynick, Zachariou, & Picciotto, 2005).

There is a comparatively larger expression of GALR1 than GALR2 mRNA in the LC (O'Donnell, et al., 1999). Investigators have identified [^{125}I]GAL binding at all levels of the LC and the region medial to the LC (containing LC denrites), an effect completely blocked by the addition of excess unlabeled GAL (Pieribone, et al., 1995). Additionally, somatic and dendritic GAL immunoreactivity was identified using electron microscopy (Pieribone, et al., 1995). GAL may serve to modulate LC activity via this somatic and denritic release (Xu, et al., 1998). Mice that overexpress GAL mRNA in the LC have been found to also show an increase in GALR1 mRNA in CA1 neurons (Hohmann et al., 2003), although this finding has not been fully supported (He, et al., 2005; He et al., 2007).

GALR2-null mice spend less time in open arms and make fewer entries into the open arms, demonstrating an anxiogenic-like phenotype (Bailey, Pavlova, Rohde, Hohmann, & Crawley, 2007). Activation of GALR2 reduced glutamate-induced damage to hippcampal cultures from either wild type mice or mice with a loss-of-function mutation in the GalR2 gene; furthermore, the glutamate-induced increase in phosphorylated Akt is not observed in GAL-knockout or GALR2 mutation mice, suggesting both a GAL- and GALR2-dependent mechanism for this increase (Elliott-Hunt, Pope, Vanderplank, & Wynick, 2007). Interestingly, an increase in levels of phosphorylated ERK, but not phosphorylated Akt, was indentified in mice that overexpress GAL (Elliott-Hunt, et al., 2007); thus this activity whether via ERK or Akt, supports a trophic quality for GAL. Importantly, GALR2 activate PKC (Wang, Hashemi, Fried,

Clemmons, & Hawes, 1998), thus many trophic-associated pathways are activated by GALR2 activity.

GAL indeed is implicated in development and neurite outgrowth, as GAL regulates the survival of cholinergic neurons in the medial septum and vertical limb of the diagonal band; a substantial portion of these neurons do not survive in GAL-KO mice (O'Meara et al., 2000). GAL-KO mice also show increased cell loss in a subset of DRG neurons and a substantially longer axonal regeneration following crush injury to sciatic nerve cells (Holmes et al., 2000). Mice lacking GALR1 do not show a deficit in outgrowth (Hobson, et al., 2008), and a GALR1 antagonist did not have an effect on outgrowth (Mahoney et al., 2003). However, an GALR2 agonist produced neurite outgrowth in a similar magnitude as GAL, and this effect was blocked when PKC was blocked (Mahoney, et al., 2003); hence the trophic quality of GAL is likely based on its effects at the GALR2.

Recently, a novel peptide agonist for the GALR2 has been identified, M1145, which demonstrates strong selectivity for the GALR2 subtype (90 times affinity for GALR1, and 76 times affinity for GALR2) (Runesson, Saar, Lundström, Järv, & Langel, 2009), which will allow for further characterization of GALR2, separate from the effects of GALR3. GAL(2-11), a widely used GALR agonist, demonstrates selectivity for both the GALR2 and GALR3 subtypes (Hobson, et al., 2008).

GAL also participates in the modulation of hyperexcitability in the hippocampus, an effect extending in part from hyperpolarization via activation of potassium channels by GAL (Dunne, et al., 1989). Both GALR1 and GALR2 participate in reducing the severity of hippocampal damage extending from seizure, with GALR1 more involved in the initiation of seizure and GALR2 implicated in mediating seizure progression (Mazarati & Lu, 2005).

Bilateral hippcampal injection of GAL reduced the severity of picrotoxin-induced seizures (Mazarati, Halászi, & Telegdy, 1992), and antagonists for GAL block this protective effect under perforant path stimulation or pilocarpine administration (Mazarati et al., 1998).

GALKO mice demonstrate more severe seizures, and GALOE show protection in three hyperexcitability models: perforant path stimulation, administration of kainic acid, or pentyleneterazol administration (Mazarati et al., 2000). The GALKO and GALOE mice also demonstrated, respectively, an increase and decrease in glutamate release from hippcampal slices, as well as commensurate effects in LTP induction (Mazarati, et al., 2000). Galnon, a nonpeptide GAL agonist, demonstrates strong reductions in seizure severity, increased seizure latency, and an inhibition of the proconvulsant effects of M35 (Saar et al., 2002), a potent GALR antagonist (Wiesenfeld-Hallin et al., 1992). The actions of Galnon did not occur in mice that received peptide nucleic acid antisense for GALR1 (Saar, et al., 2002), suggesting a very important role for GALR1 in anticonvulsion.

Influence of Exercise on the Locus Coeruleus and Galanin

The rate of NE turnover is increased in the brains of running rats (Gordon, Spector, Sjoerdsma, & Udenfriend, 1966); treadmill running also enhances extraneuronal metabolism of NE in the hippocampus as metabolite (MHPG) levels, but not NE levels, were higher in rats that ran on treadmills for 8 weeks compared to sedentary animals; an increase not observed in rats with voluntary access to wheels (Dunn, Reigle, Youngstedt, Armstrong, & Dishman, 1996). Short-term treadmill running appears to have an age-related effect on TH mRNA in the LC however, with 5 days of 30 to 60 minutes of daily running producing an increase in LC TH mRNA of 4 month old rats, but not 24 month old rats (Tümer, et al., 2001).

As previously mentioned, stress is involved in response to treadmill running, so these effects could be attributed to a stress effect. Another study, with six weeks of treadmill running, did not produce the same effect on LC TH expression (O'Neal, et al., 2001), which appears to be unaffected by chronic voluntary activity wheel running (Soares, et al., 1999). Interpreting forced running treadmill data is considerably trickier than data generated from voluntary running, as forced wheel running dramatically influences CRH neuronal activation in the PVN, compared to spontaneous wheel running (Yanagita, et al., 2007). This stress effect is also observed with forced treadmill running (Timofeeva, et al., 2003). Considering that the LC receives projections from the dorsal cap of the PVN (Aston-Jones, et al., 1986), and also that CRH modulates tonic LC activity (Valentino & Foote, 1988), studies seeking to characterize LC activity should avoid stress effects unless they are under study. Rats run roughly the same distances with free access to an activity wheel, compared to treadmill running (Yanagita, et al., 2007).

Wheel running reduces foot shock escape latency and protects against the depletion in LC NE that is associated with foot shock (Dishman, et al., 1997). In mice, wheel running produced effects on the forced swim test, learned helplessness, and tail suspension models similar to amitryptilline and desipramine administration, both potent 5-HT and NE reuptake inhibitors (Duman, et al., 2008). A clomipramine depression model study, comparing three conditions, including imipramine administration, treadmill running, and wheel running, showed that increased NE amounts in the frontal cortex of rats occurred in all groups, while wheel running and imipramine administration reduced frontal cortex beta noradrenergic receptor binding (Yoo, et al., 2000). While this effect could be attributed in part to the stress of treadmill running, only rats in the wheel running groups exhibited greater frequency of ejaculation, and wheel runners

and concaminant wheel running and imipramine administration both significantly increased intromission frequency as well (Yoo, et al., 2000).

GAL mRNA levels are significantly increased in the LC following treadmill running (O'Neal, et al., 2001). Activity wheel running also induces an increase (Holmes, et al., 2006), an effect not influenced by a β-adrenoreceptor antagonist (Van Hoomissen, et al., 2004). LC GAL mRNA is also strongly correlated (r=0.593) with mean distance on running wheels in rats (Eisenstein & Holmes, 2007; Holmes, et al., 2006).

When considering that wheel running induces beneficial changes in GAL functioning, it is important to understand the role that capacity for running may play in GAL modulation. Rats can be selected over generations to demonstrate greater or lesser capacity for aerobic exercise, measured by treadmill running to exhaustion (Koch & Britton, 2001, 2005, 2008). By selecting rats in each generation for high or low capacity for this measurement and breeding them, later generations are produced that demonstrate dramatic differences in ability to run to exhaustion (Koch & Britton, 2001, 2008). These effects may be related to differences in maximal oxygen uptake (Gonzalez, et al., 2006) and peripheral muscle processes, as gastrocnemius of HCR show more capillaries per unit than those of LCR (Howlett, et al., 2002), which contributes to a greater capacity of HCR to deliver and utilize oxygen in skeletal muscle (Howlett, et al., 2009). HCR and LCR rats also exhibit differences in corticosterone and striatal dopamine activity after wheel running (Waters et al., 2008), and dopamine and serotonin autoreceptor activity (Foley et al., 2006). These rats provide a unique model to explore the role that aerobic capacity plays in any exercise-induced neurophysiological change.

CHAPTER 3

VOLUNTARY WHEEL RUNNING AND EEG MEASURES OF KAINIC ACID-INDUCED SEIZURES IN RATS[1]

[1] Murray, P. S., Spradley, J. M., Dishman, R. K., and Holmes, P.V. Submitted to *Behavioural Brain Research* on 4/14/2010.

Abstract

Voluntary wheel running has previously been shown to protect against behavioral seizures induced by kainic acid. This phenomenon was further examined in the present study with EEG measures in exercising and sedentary rats following administration of a convulsant dose of kainic acid. Male Sprague-Dawley rats were provided with free access to running wheels (exercise condition) or no running wheels (sedentary condition). Following three weeks of these conditions, epidural EEG electrodes were implanted into each rat and a two-channel EEG platform was affixed to the skull. Two days following surgery rats received systemic 10 mg/kg kainic acid injections. EEG measurements were recorded and the spike-wave incidence was quantified. A seizure severity index derived from the Racine scale was used for behavioral seizure ratings. Spike-wave discharges and seizure severity ratings were highly correlated, $rho = 0.772$, $p < 0.05$. The difference in spike-wave discharges between conditions approached statistical significance, $t(6) = 1.72$, $p = 0.068$. These results suggest that voluntary wheel running may lead to reduced seizure severity, as measured via both spike-wave quantification and behavioral seizure rating. Furthermore it was established that spike-wave quantification is a simple and convenient EEG measure of seizure severity in that it was strongly associated with behavioral seizure rating. Thus, the present study lays the foundation for future application of EEG techniques to study the neurobiological mechanism for the anticonvulsant effects of chronic voluntary exercise.

Keywords: EEG, exercise, kainic acid, anti-convulsant, seizure, spike-wave discharge

Introduction

Exercise produces a variety of beneficial effects at behavioral and neurobiological levels. Exercise thus reduces depression symptoms in humans (Dunn, et al., 2005; Dunn, Trivedi, & O'Neal, 2001) and leads to reduced escape latencies in learned helplessness models of depression (Dishman, et al., 1997; Duman, et al., 2008; Greenwood, et al., 2003). Exercise mitigates the harmful effects of chronic stress (Zheng et al., 2006), oxidative stress (Radak et al., 2001), and stroke (Ding, et al., 2004), and it attenuates the impact of aging on learning (van Praag, et al., 2005). Running on a cage wheel lessens the severity of seizures (Reiss, et al., 2009) while treadmill running has been shown to reduce seizure recurrence (Arida, et al., 1999) and hippocampal hyperexcitability (Arida, et al., 2004). These latter protective effects may be related to an enhancement of galanin (GAL) activity, a neuropeptide with neuroprotective properities in conditions of hyperexcitability (Elliott-Hunt et al., 2004).

As previously demonstrated in this laboratory, regular exercise increases GAL mRNA in the locus coeruleus (LC) (Holmes, et al., 2006; O'Neal, et al., 2001; Van Hoomissen, et al., 2004). GAL coexists with norepinephrine in the majority of noradrenergic cell bodies of the LC (Holets, et al., 1988); these GALergic fibers project throughout the forebrain, including both neocortex and hippocampus (Melander, et al., 1986a); the cell bodies of these fibers originate largely from the central and dorsal portions of the LC (Holets, et al., 1988), leave the structure mainly via the dorsal bundle, and enter the hippocampus through both dorsal (via supracallosal striae) and ventral pathways (Melander, Staines, & Rökaeus, 1986b).

Using GAL under- and over-expressing transgenic mice, Elliott-Hunt et al. (Elliott-Hunt, et al., 2004) established that GAL protects against an array of excitatory insults in hippocampal cells, including kainic acid (KA), a potent agonist for the eponymous ionotropic kainaite receptor

(for review see Bettler & Mulle, 1995). Systemic administration of KA reliably leads to limbic status epilepticus and characteristic hippocampal cell loss (Ben-Ari, Tremblay, Ottersen, & Meldrum, 1980; Sperk, 1994), and represents a useful experimental model of temporal lobe epilepsy (Sperk, 1994). GAL has consistently been shown to exert anticonvulsant effects in a variety of animal models of epilepsy (Mazarati, 2004; Mazarati, et al., 1992; Mazarati, et al., 2000; Mazarati, Langel, & Bartfai, 2001; Mazarati, et al., 1998; Mazarati & Lu, 2005); these findings support the hypothesis that exercise, at least in part via upregulation of endogenous GAL, would exert neuroprotective and consequently anticonvulsant effects.

Spike-wave discharges (SWDs) are electroencephalographic (EEG) paroxysms that are associated with absence seizures (Drinkenburg, Schuurmans, Coenen, Vossen, & van Luijtelaar, 2003; Paz, Chavez, Saillet, Deniau, & Charpier, 2007). SWD morphology is readily observable as complexes of sharp spikes and slow waves (Van Luijtelaar & Coenen, 1986) and, when appropriately defined, SWDs can be quantified by frequency of occurrence. SWDs thus provide a relatively simple and quantitative EEG measure of seizures that can be reasonably characterized even in conditions of excessive EEG noise.

This study sought to describe the difference in SWD EEG activity in exercising and sedentary rats immediately following KA administration, characterize the association between behavioral seizure rating and SWDs, and examine the association between behavioral seizure rating and condition. It was expected that SWDs would be associated with seizure ratings, thereby verifying that this measure serves as a reliable indicator of seizure severity. Additionally it was predicted that sedentary rats, compared to exercising rats, would express more severe seizures in response to KA, as measured by a greater incidence of SWDs and higher behavioral seizure ratings.

Method

Animals.

Male Sprague-Dawley rats (n = 8, 2 months old) were individually housed in polycarbonate cages in a temperature and humidity-controlled environment that was maintained on a 12 hour light-dark schedule, with *ad libitum* access to food and water. Following acclimatization, animals were randomly assigned to one of two groups: exercise or sedentary.

Exercise Protocol.

Each rat in the exercise condition was provided with a stainless-steel activity wheel (diameter 33.5 cm) connected to an electromagnetic revolution counter. The rats had free access to the wheels for 21 days. The daily running distance was recorded and determined by multiplying the number of revolutions by the circumference of the wheel (1.05 m).

EEG.

Equipment and Recording.

Instrumentation, all manufactured by Pinnacle Technology, Inc. (Lawrence, Kansas), consisted of the following items: a head-mounted platform (8.467 mm x 8.467 mm) with 2-channel EEG and wire leads, preamplifier, commutator and mounting plate, a data acquisition and conditioning system (DACS), and a Dell Latitude computer interfaced with the DACS.

EEG recordings (sampled at 1000 Hz) were initiated a minimum of 10 minutes prior to and 120 minutes following injection.

Electrode Implantation Surgery.

Rats were anesthetized with isoflurane delivered by a vaporizer via a nosecone. A rostral-caudal midline incision was made from between the eyes to behind the ears. The skull surface was cleaned of connective tissue to lateral ridges and allowed to dry in order to maximize

adhesion of the head-mounted platform. The platform was affixed to the skull surface using cyanoacrylate adhesive, centered on the midline with the rostral-facing edge approximately 1 mm anterior to bregma. Epidural holes were drilled immediately to the rostral-facing edge of the corners. Screws were installed using a jeweler's screwdriver; the corresponding EEG leads were wrapped around each screw. This procedure was repeated for the caudal-facing edge of the platform corners, with posterior screws and EEG leads installed. The wounds were sutured and the rats were maintained in a warm cage until recovery.

Seizure Induction and Characterization.

KA Administration and Behavioral Rating.

On the second day following surgery, rats received intraperitoneal injections of 10 mg/kg KA.

Behavioral seizure ratings were determined using a classification system developed by Racine (Racine, 1972), slightly modified (Hoffman, Moore, Fiskum, & Murphy, 2003; Lothman & Collins, 1981). These ratings are based on seizure-typical behaviors: 0 = no seizure behavior, 1 = minor behaviors including catatonia, wet dog shakes, and headbobbing, 2 = minor behaviors including chewing, salivation, and rearing without loss of balance, 3 = chewing, salivation, and rearing with loss of balance, 4 = tonic-clonic seizures, and 5 = death.

SWD.

SWDs were defined as large spikes (amplitude at least twice that of background EEG activity) followed by slow waves (Drinkenburg, et al., 2003; Van Luijtelaar & Coenen, 1986). To be counted, each SWD lasted a minimum of 1 second and was identified by shape (figures 3.1 and 3.2). To qualify as independent SWDs, there had to be a minimum of 1 second between large spikes. With the negative deflection of the spikes, SWD morphology resembled type 2

SWDs (Sitnikova & van Luijtelaar, 2007; Van Luijtelaar & Coenen, 1986) due to the placement of the electrode over the left occipital cortical area (Sitnikova & van Luijtelaar, 2007).

Data Analysis.

A one-way Students's *t* test was performed for the SWD data. The phi coefficient was calculated to characterize the association between seizure severity (moderate or severe) and exercise condition (exercise or sedentary); the phi coefficient provides a measure of association for two binary variables (Guilford, 1941). The nonparametric Spearman's rho was calculated to characterize the association between SWD and seizure severity ratings. GPower 3.0.10 (Düsseldorf, Germany) was used to calculate effect size (Faul, Erdfelder, Lang, & Buchner, 2007), and SPSS 13.0 (Chicago, Illinois) was used for all other analyses.

Results

In the interest of reducing type I error, all possible SWD complexes were included in the tally, minus movement artifacts and high-amplitude activity associated with full seizures. Counting was conducted blind of subject ID or condition. For the sedentary condition: M = 223.5, SD = 74.02, and for exercise: M = 157.0, SD = 22.33. The difference in the number of SWDs between the groups was not significant, $t(6) = 1.72$, $p = 0.068$. The effect size was large, $d = 1.22$.

To assess the relationship between seizure rating and SWD, Spearman's rho was calculated. A strong positive correlation between seizure severity and SWD incidence was identified, with $rho = 0.772$, $p = 0.025$.

Seizure severity ratings were organized into a contingency table (table 3.1). Seizure severity was structured simply as moderate (potential score of 1, 2 or 3), or severe (potential

score of 4 or 5). No rat scored 1 or 5 on the scale. A phi coefficient was calculated and no significant association was identified, *phi* = 0.577, $p = 0.051$.

Discussion

SWD incidence was strongly correlated with seizure severity rating, verifying that a discrete tally of the number of SWDs represents a reliable index of seizure severity.

Exercising rats exhibited fewer SWDs than sedentary rats, though this difference was not significant at our established alpha of 0.05. Considering the large effect size, the difference may be considered marginally significant ($p = 0.068$) and consistent with our previous findings of greater seizures severity in sedentary rats (Reiss, et al., 2009). Additionally, although exercise condition was not significantly associated with seizure rating ($p = 0.051$), it was close; with a strong *phi* = 0.577 and no seizure ratings in excess of 3 in the exercise group, the likelihood of a relationship should certainly not be dismissed. Thus, over both of our measures of seizure behavior, exercising rats showed a marginal, but readily observable, reduction in kainic acid response compared to sedentary rats.

Although voluntary wheel running reduces the recurrence of spontaneous seizures using pilocarpine as an induction agent (Arida, et al., 2009) and the severity of behavioral seizures (Reiss, et al., 2009), running has been associated with a increased vulnerability of hippocampal neurons to the effects of intrahippocampal KA administration (Ramsden, Berchtold, Patrick Kesslak, Cotman, & Pike, 2003). Underlying this phenomenon could be the simultaneous running-induced enhancement of trophic factor activity in the hippocampus, such as brain-derived neurotrophic factor (Van Hoomissen, et al., 2003), and the associated upregulation in NMDA receptor activity (Farmer, et al., 2004). However, the effect of GAL to reduce excitotoxic damage (Elliott-Hunt, et al., 2004; Elliott-Hunt, et al., 2007) and its associated

anticonvulsant properties (Lerner, Sankar, & Mazarati, 2008; Mazarati, 2004; Mazarati, et al., 1992; Mazarati, et al., 2000; Mazarati, et al., 2001; Mazarati, et al., 1998; Mazarati & Lu, 2005) works largely to counteract the potential for this to manifest as an increase excitotoxic vulnerability. Any effects in the present study were not due to systemic pharmacokinetic factors, since anticonvulsant effects of exercise are observed following intracerebroventricular administration of KA, after 3 weeks of voluntary wheel running (Reiss, et al., 2009). Additionally, Gobbo and O'Mara (Gobbo & O'Mara, 2005) found that exercise prior to KA-induced neurodegeneration led to improved performance on learning tasks.

EEG provided a valid index of subthreshold seizure activity; the SWD morphology was readily identified and easily quantified using a systematic, blind, reasonably liberal classification. This method showed a strong correlation with behavioral seizure ratings, establishing that a simple tally of SWD phenomena over a discrete time period provides a legitimate indicator of seizure severity.

Conclusion.

Although seizure ratings and SWD incidence were not significantly associated with exercise condition, sedentary rats did appear to experience more severe seizures, a difference that may have borne statistical significance in a study of larger group sizes. Importantly, we verified that SWD frequency is related to the seizure-severity rating index we utilized, indicating a useful measure of seizure severity.

Based on the potential for differences in SWD frequency that we have identified as well as the potential for association between exercise condition and seizure rating, our results provide additional basis for further inquiry into the neuroprotective nature of exercise, especially in conditions sensitive to hyperexcitability such as epilepsy.

Table 3.1 Seizure severity for all rats.

Seizure Severity Contingency Table

	Seizure Severity		
	Moderate	High	Total
Exercise	4	0	4
Sedentary	2	2	4
Total	6	2	8

Figure 3.1 Example SWD EEG output. Sedentary Subject.

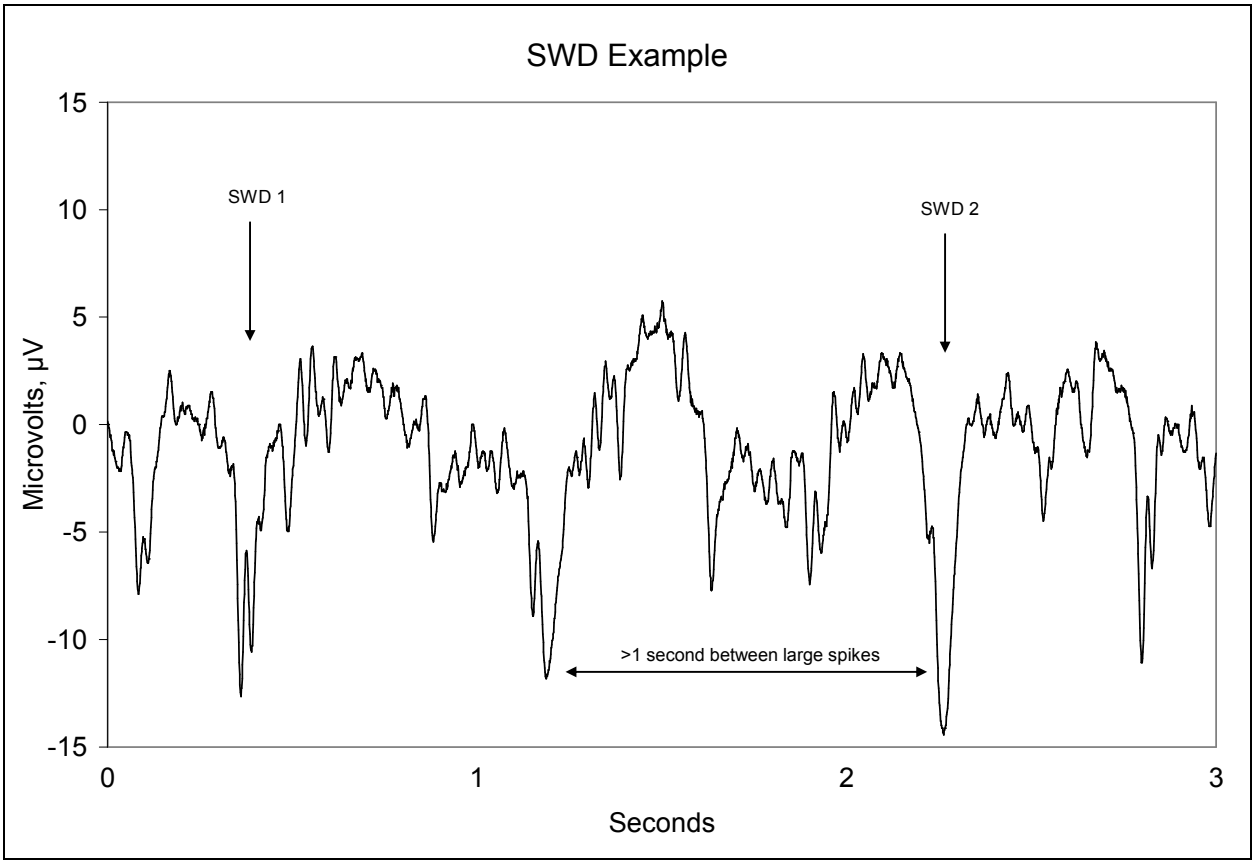
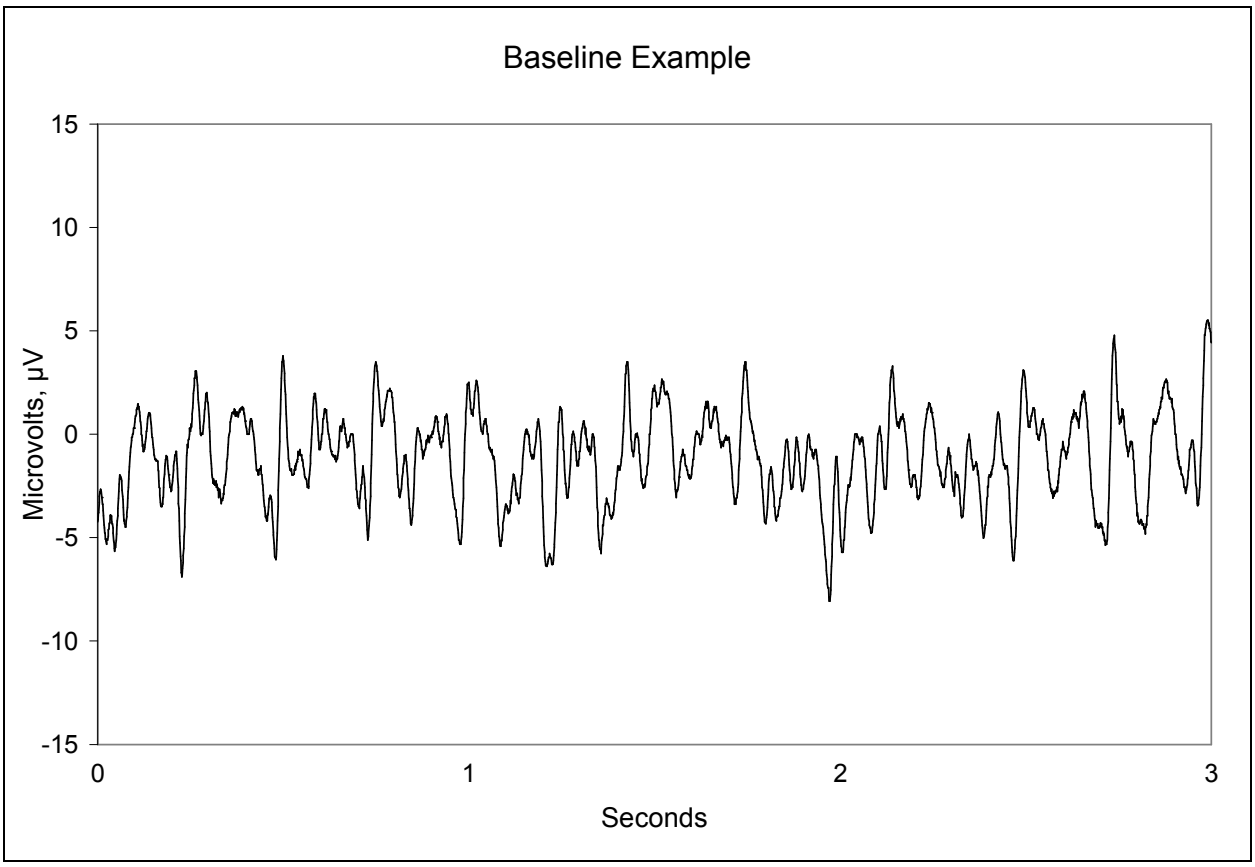

SWD Example
15
10
5
0
-5
-10
-15
Microvolts, µV
SWD 1
SWD 2
>1 second between large spikes
0
1
2
3
Seconds

Figure 3.2 Example baseline EEG output. Sedentary Subject.


Baseline Example
15
10
5
0
-5
-10
-15
Microvolts, µV
0
1
2
3
Seconds

CHAPTER 4

LOCUS COERULEUS GALANIN GENE EXPRESSION IS ENHANCED AFTER EXERCISE IN RATS SELECTIVELY BRED FOR HIGH CAPACITY FOR AEROBIC EXERCISE[1]

[1] Murray, P. S., Groves, J. L., Dishman, R. K., Pettett, B. J., Britton, S. L., Koch, L. G., and Holmes, P. V. To be submitted to *Peptides*.

Abstract

The neuropeptide galanin extensively coexists with norepinephrine in locus coeruleus (LC) neurons. Previous research in this laboratory has demonstrated that unlimited access to activity wheels in the home cage increases mRNA for galanin (GAL) in the LC, and that GAL mediates some of the beneficial effects of exercise on brain function. To assess whether aerobic running capacity modulates this upregulation in galanin mRNA, strains of rats selectively bred for either high (HCR) or low capacity (LCR) for aerobic exercise and Sprague-Dawley (SD) rats were provided with free access to running wheels or no wheels for three weeks. Following this exercise protocol, mRNA for tyrosine hydroxylase (TH) and GAL were measured in the LC. The difference in running distances between the rat strains was significant, and age contributed as a significant covariate. Both strain and exercise condition significantly affected GAL mRNA expression, but not TH mRNA expression. GAL was elevated in exercising HCR and SD rats compared to the LCR rats. Overall running distance significantly correlated with GAL mRNA expression, but not with TH mRNA expression. No strain differences in GAL or TH gene expression were observed in sedentary rats. Thus, intrinsic aerobic running capacity influences GAL gene expression in the LC only insofar as actual running behavior is concerned; aerobic capacity does not influence GAL expression beyond the changes associated with running.

Keywords: galanin, tyrosine hydroxylase, aerobic capacity, running-wheel

Introduction

Chronic exercise influences brain function in a variety of beneficial ways. Exercise reduces symptoms of depression and anxiety in humans (Dunn, et al., 2001) and animal models (Duman, et al., 2008), mitigates the harmful effects of stroke (Ding, et al., 2004), and also modulates the severity of seizure effects (Elliott-Hunt, et al., 2004; Mazarati, et al., 2000; Reiss, et al., 2009). Voluntary wheel running enhances performance on tests of spatial learning in rats (van Praag, et al., 1999) and reduces the negative impact of aging on this performance (van Praag, et al., 2005). Many of these effects may be attributed to adaptations in intracellular signaling. Exercise is associated with activation of survival and proliferation pathways in the hippocampus, such as PI3-kinase (Chen & Russo-Neustadt, 2005), MAPK (Shen, et al., 2001), as well as increased activity of vesicle-related proteins synapsin I (Vaynman, et al., 2004) and synaptophysin (Vaynman, et al., 2006).

The recent finding that regular exercise upregulates galanin (GAL) expression in the noradrenergic locus coeruleus (LC) suggests that GAL may mediate some of the neural and behavioral effects of exercise. GAL coexists with norepinephrine (NE) in most of the cell bodies of the LC (Holets, et al., 1988; Melander, et al., 1986a). The LC comprises the primary noradrenergic innervation of the central nervous system and projects to areas throughout the forebrain including neocortex and hippocampus, as well as hypothalamus, thalamus, cerebellum and spinal cord (Loughlin, et al., 1986; Melander, et al., 1986a; Nygren & Olson, 1977). Receptors for GAL are distributed throughout the brain, with particularly high densities found in the hippocampus and diencephalon, as well as the LC itself (O'Donnell, et al., 1999). The GAL receptor subtypes GALR1 and GALR2 are G-protein coupled and linked to adenylyl cyclase inhibition (He, et al., 2005) and activation of potassium channels (Dunne, et al., 1989). GAL

receptor activity is associated with pathways such as Akt, ERK (Elliott-Hunt, et al., 2007), or PKC (Wang, et al., 1998). Both treadmill running and voluntary wheel running increases GAL mRNA in the LC (Holmes, et al., 2006; O'Neal, et al., 2001; Van Hoomissen, et al., 2004).

The present experiments investigated whether intrinsic running capacity influences exercise-induced upregulation of GAL. Rats have been selectively bred to express greater or lesser intrinsic aerobic capacity than normal (Koch & Britton, 2001, 2005, 2008). This selection process yields later generations that demonstrate a substantial expansion or reduction in capacity to run to exhaustion on a treadmill relative to earlier generations. Thus, the high capacity and low capacity rat strains (HCR and LCR, respectively) developed by Koch and Britton (Koch & Britton, 2001) differ in their capacity to run on a treadmill to the point of exhaustion by several fold (Koch & Britton, 2008). These differences may be associated with a greater capacity of HCR to deliver and utilize O_2 in skeletal muscle compared to LCR rats (Howlett, et al., 2009).

Since age is another factor that influences exercise effects in rats (Adlard, et al., 2005), the present experiments included a range of ages, which was analyzed as a covariate. The present studies thus aimed to further characterize the nature of the relationship between wheel running and GAL in the LC, and address the following questions: Does intrinsic aerobic running capacity influence the exercise-induced upregulation of LC GAL? What effect does wheel running have on mRNA for tyrosine hydroxylase (TH), the rate limiting enzyme in NE production (Nagatsu, et al., 1964)? Does age covary with the effect of exercise on LC GAL (or potentially TH) mRNA upregulation?

Method

Subjects.

Previous work details the development of a rat model of aerobic running capacity (Koch & Britton, 2001). Briefly, rats chosen from a genetically heterogeneous N:NIH founder population ran to exhaustion on a treadmill, and the distances were recorded. The 13 lowest running males and 13 lowest running females comprised the beginning of a LCR line, while the 13 highest running males and 13 highest running females started an HCR line (Koch & Britton, 2001). At 10 generations, there was an overall difference in running capacity between HCR and LCR rats of 317% (Koch & Britton, 2005); by 21 generations, the difference reached 450% (Koch & Britton, 2008).

A total of 84 rats, HCR, LCR, and Sprague-Dawley (SD), were included. HCR and LCR rats were shipped from the University of Michigan, and Sprague-Dawley rats were supplied by Harlan. Ages ranged from 60 days to 321 days.

Exercise Protocol.

Rats in the exercise condition were housed individually in polycarbonate cages and provided with unlimited access to a stainless steel cage wheel for 21 days. Revolutions were recorded with a magnetic counter. Running distances were recorded every other day and calculated by multiplying revolutions by the circumference of the wheel (1.05 m). 4 rats were excluded from analysis because mean daily revolutions did not exceed 50, which was likely associated with their age and weight. Running data from a total of 46 rats were analyzed. Animals in the sedentary condition were housed under identical conditions, without a cage wheel.

In Situ Hybridization.

Following completion of the exercise protocol, rats were decapitated and the brains were removed and frozen using dry ice, then stored at -80^{o}C. Coronal sections at the level of LC were cut using a Microm cryostat (Waldorf, Germany) at a thickness of 12 μm. Sections were thaw-mounted onto gelatin-coated glass microscope slides. Anatomical location was verified using a 0.1% thoinin stain. Sections were fixed in 4% (v/v) formaldehyde in 0.12 M sodium phosphate-buffered saline (PBS) solution, rinsed in PBS, and soaked in 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine HCl-0.9% (v/v) NaCl. Sections were dehydrated in a series of ethanol washes, delipidated in chloroform, and rinsed in ethanol.

Oligonucleotide probes (Oligos Etc., Wilsonville, Oregon) for GAL and TH, were labeled at the 3' end with [35S]-dATP (New England Nuclear, Boston, Massachusetts), terminal deoxynucleotidyl transferase (TdT, 25 units/ml; Roche, Indianapolis, Indiana), and tailing buffer. Column separation was used to remove unbound nucleotide. Sections were hybridized with the radiolabeled probes in solutions containing 25% (v/v) formamide, 72 mM NaCl, 3.2 mM Tris-HCl, 0.0032 mM EDTA, 0.001% (v/v) sodium pyrophosphate, 0.004% (v/v) sodium dodecyl sulfate, 0.002 mg/ml heparin sulfate, and 2% (v/v) dextran sulfate. Sections were incubated overnight at 37^{o}C, followed by a series of washes in SSC and SSC-50% formamide, water and ethanol, and then were dried.

Hybridized sections were placed into film cassettes and exposed to autoradiographic film (BioMax MR, Eastman Kodak, Rochester, NY) and developed with Kodak GBX developer and Kodak GBX fixer.

Film Analysis.

Autoradiographs were scanned into Adobe Photoshop using a high resolution scanner (Microtek, San Francisco, CA), with a PowerMAC G4 (Apple, Inc., Cupertino, CA) for processing. ImageJ (National Institutes of Health, Bethesda, MD) was used to highlight and quantify grayscale brightness units in the LC.

Statistical Analysis.

For all analysis of covariance (ANCOVA) calculations, age was included as a covariate. A repeated-measures ANCOVA with Greenhouse-Geisser correction was performed to examine running distance over time. ANCOVAs were used to identify any differences in grayscale units for GAL mRNA or TH mRNA among the groups. For multiple comparisons, the Bonferonni adjustment was used. To determine whether a relationship existed between either mRNA for GAL or TH and overall running distance, Pearson correlation coefficients were calculated. SPSS 13.0 (Chicago, IL) was used for all statistical analyses.

Results

Running Distance.

Overall, daily running distance increased over time, $F(1.909, 80.178)=20.773$, $p<0.01$. Age contributed significantly as a covariate to differences in running $F(1, 42)=16.440$, $p<0.01$, and the strains exhibited differences in running distance, $F(2, 42)=6.256$, $p<0.01$. HCR rats ran more than LCR rats, $p<0.01$, but HCR rats did not differ from SD rats, $p=1.0$. SD rats also ran more than LCR rats, $p=0.013$ (figure 4.1). Significant interactions were identified between the covariate age and running over time, $F(1.909, 80.178)=10.771$, $p<0.01$, and also between strain and running over time, $F(3.818, 80.178)=4.253$, $p<0.01$.

LC mRNA Expression.

GAL.

The omnibus F test for our ANCOVA of LC GAL mRNA was statistically significant, $F(6, 77)=3.872$, $p<0.01$. While age did not significantly contribute to differences in GAL mRNA $F(1, 77)=2.959$, $p=0.089$, selection (HCR, LCR, or SD) did, $F(2, 77)=7.273$, $p<0.01$, as did exercise condition, $F(1, 77)=5.365$, $p=0.023$. However, selection and exercise condition did not interact to influence LC GAL mRNA, $F(2, 77)=1.117$, $p=0.332$. The LCs of HCR showed greater GAL mRNA than than those of LCR, $p<0.01$, and SD showed greater LC GAL mRNA than did LCR, $p<0.01$, but no significant difference was identified between SD sections and HCR sections, $p=1.0$.

Exercise did produce a significant effect, with exercising rats showing greater LC GAL mRNA expression than sedentary rats, $p=0.023$. Overall running distance and GAL mRNA expression were significantly correlated, $p=0.028$, $r=0.317$. For a scatterplot of the data, see figure 4.2. See also figures 4.3 and 4.4.

TH.

The omnibus F test of TH mRNA was not significant, $F(6, 54)=0.659$, $p=0.683$, indicating that strain and exercise condition did not significantly differ or interact with regard to TH mRNA expression in the LC (figure 4.5).

Overall running distance and TH mRNA expression were not significantly correlated, $r=0.285$, $p=0.079$. See figure 4.6.

Discussion

In the present study, we established that aerobic running capacity influences exercise-induced upregulation of GAL in the LC. The SD group ran with total distance values having

fallen in between the other groups, though nearer to HCR than LCR (figures 4.1 and 4.3); so it is reasonable to conclude based on the present data that both above-average or average running capacities facilitate an upregulation of GAL in the LC in a similar manner following voluntary exercise. However, a below-average aerobic running capacity appears to influence LC GAL regulation in a negative manner (albeit with a nonsignificant interaction), with LCR rats showing little GAL mRNA difference between exercise and sedentary groups (figure 4.4).

Forced exercise, in the form of short-term treadmill running, has been shown to have an age-related effect on LC TH mRNA expression, with younger rats showing a response to five daily bouts of treadmill running, but not older rats (Tümer, et al., 2001). Though the effects of forced exercise paradigms may be difficult to interpret because they involve stress, both voluntary wheel running and treadmill running have been shown to protect against stress-related depletion in LC NE (Dishman, Renner, White-Welkley, Burke, & Bunnell, 2000; Dishman, et al., 1997), so exercise likely influences NE activity in the LC in some fashion. However, in agreement with previous work (Soares, et al., 1999), wheel running did not produce an effect on TH expression in the LC (figure 4.5), a finding previously observed in this laboratory with treadmill running (O'Neal, et al., 2001).

It is important to understand that while exercise enhances GAL activity, age may factor into its upregulation. Indeed, GAL expression changes over a lifespan; septal GAL protein is reduced in older rats (De Bilbao, Jazat, Lamour, & Senut, 1991), while GAL binding sites are increased in piriform and entorhinal corticies, and dentate gyrus in older rats (Krzywkowski, Lagny-Pourmir, Jazat, Lamour, & Epelbaum, 1994). Other neuronal factors that exhibit a robust increase with exercise, such as brain-derived neurotrophic factor, show a diminished exercise effect with age (Adlard, et al., 2005).

The HCR and LCR rats demonstrated a dramatic difference in running, with HCR rats running upwards of 2500 revolutions (2625 m) on average per day more than LCR rats. Interestingly, SD running behavior fell closer to the daily running distance of HCR rats. On average HCR rats ran, total distance, more than six times as much as LCR rats (figure 4.3). This proportion (approximately 650%), is larger than the proportional difference in distance to exhaustion of 450% observed at generation 21, which may be in line with further selection effects as the HCR/LCR rats in this study are of approximately generation 23. The interactions identified in the repeated measures ANCOVA verified what seems apparent in the running data; HCR rats increase their running behavior more over three weeks of running than do the LCR rats. Also, rats increase their running behavior less over time with age. These findings further highlight the profound effects selection has had on aerobic running capacity, and help to define how the running behavior of these selected rats relates to the running behavior of the more commonly used SD rat, which is the strain employed for most of the previous exercise research in this laboratory.

Exercise condition and selection did not significantly interact to produce an effect on GAL mRNA expression, but overall running did significantly correlate with GAL mRNA expression. More running did generally did correspond with more GAL expression. Considering these relationships, it is then likely that running capacity due to selection affects GAL mRNA expression in the LC only insofar as actual running behavior is increased, without any additional synergistic influence.

Conclusion.

Selection for aerobic running capacity produces dramatic differences in running behavior, and age contributes to this difference in behavior. Intrinsic aerobic running capacity does

influence GAL mRNA expression in the LC, with exercising HCR and SD rats exhibiting higher GAL mRNA than their sedentary counterparts. No such differences between exercising and sedentary groups were seen in the LCR, which may be explained by their relative lack of running since total running distance is related to GAL upregulation in the LC. Age does not participate as a covariate in these effects. LC TH mRNA expression is not influenced by wheel running. These results further suggest that regulation of GAL in the LC is tightly coupled to exercise.

Figure 4.1 Mean two-day running distance values per group, in meters.

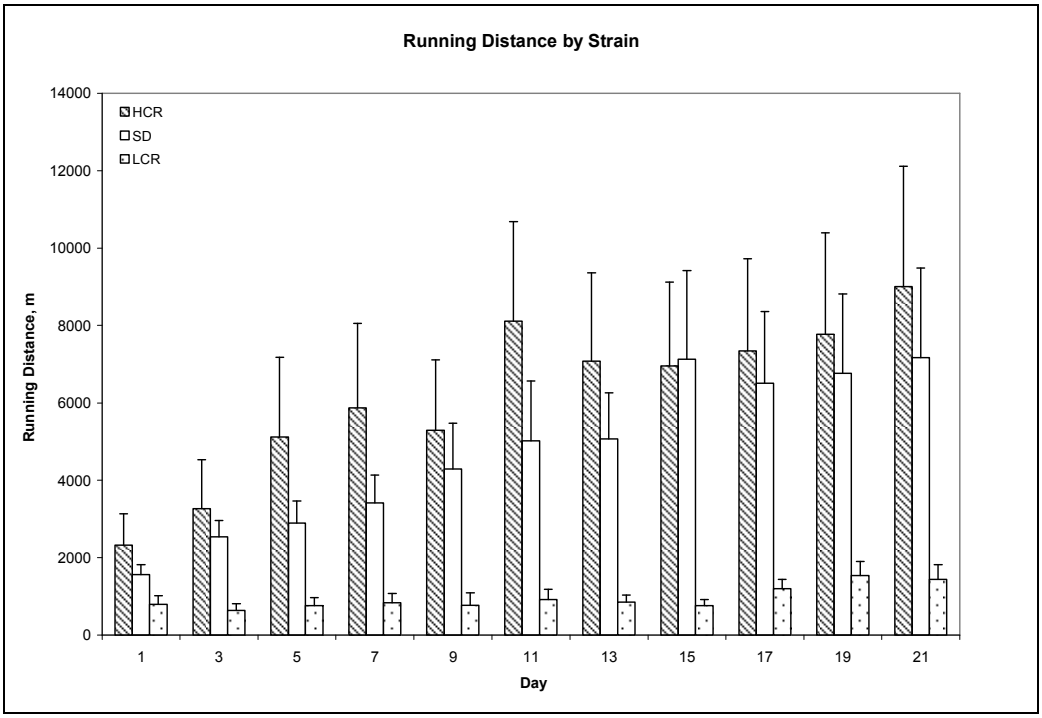

Running Distance by Strain
HCR
SD
LCR
Running Distance, m
14000
12000
10000
8000
6000
4000
2000
0
1
3
5
7
9
11
13
15
17
19
21
Day

Figure 4.2 Total running distance (in meters) vs. GAL mRNA (grayscale units).

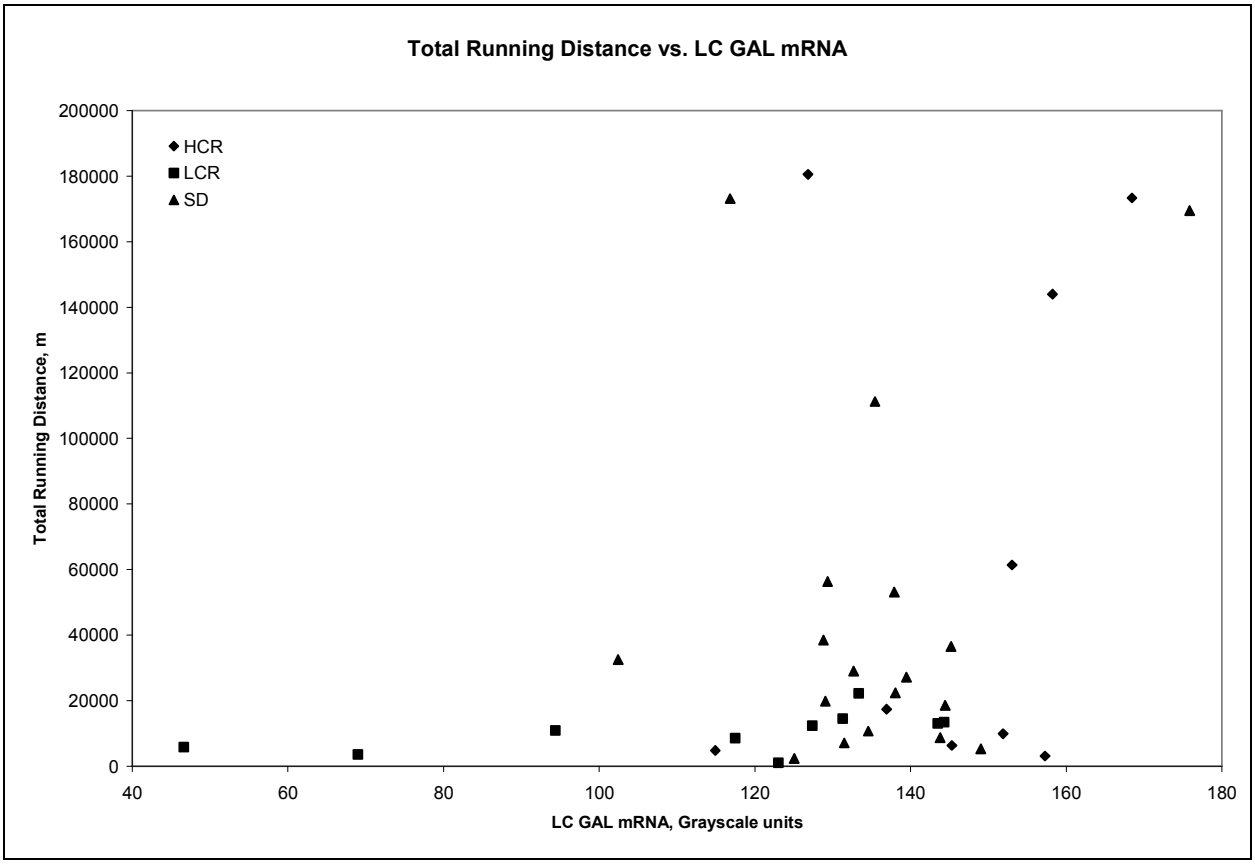

Total Running Distance vs. LC GAL mRNA
HCR
LCR
SD
Total Running Distance, m
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
40
60
80
100
120
140
160
180
LC GAL mRNA, Grayscale units

Figure 4.3 Mean total running distance values per group, in meters.

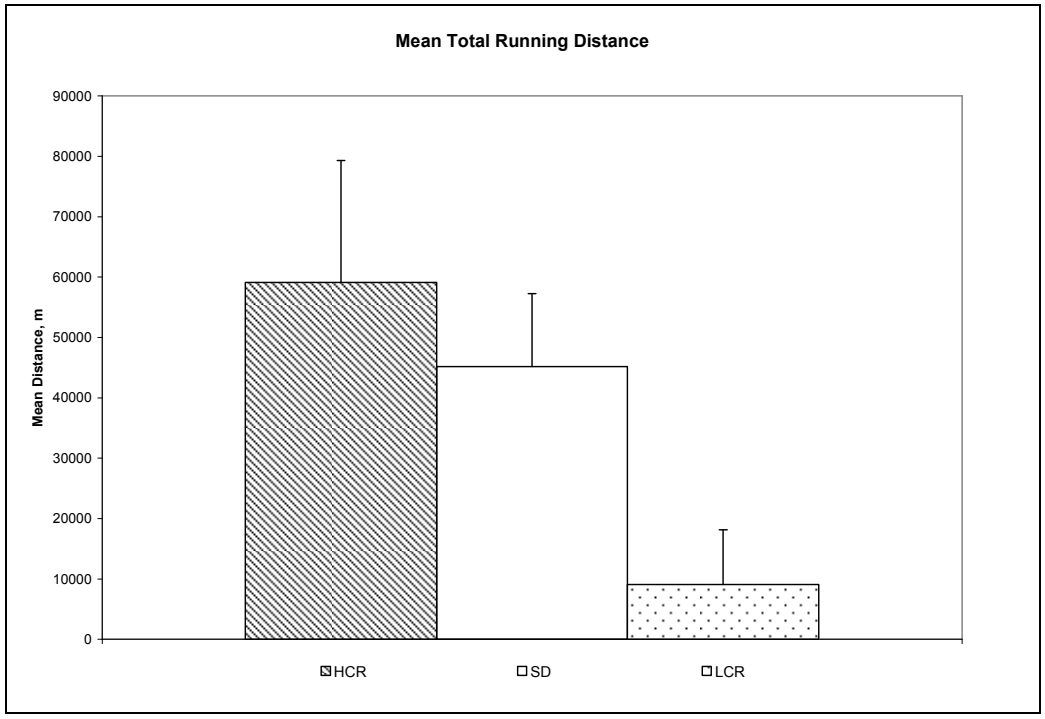

Mean Total Running Distance
Mean Distance, m
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
HCR
SD
LCR

Figure 4.4 Mean GAL mRNA expression in the LC, grayscale units

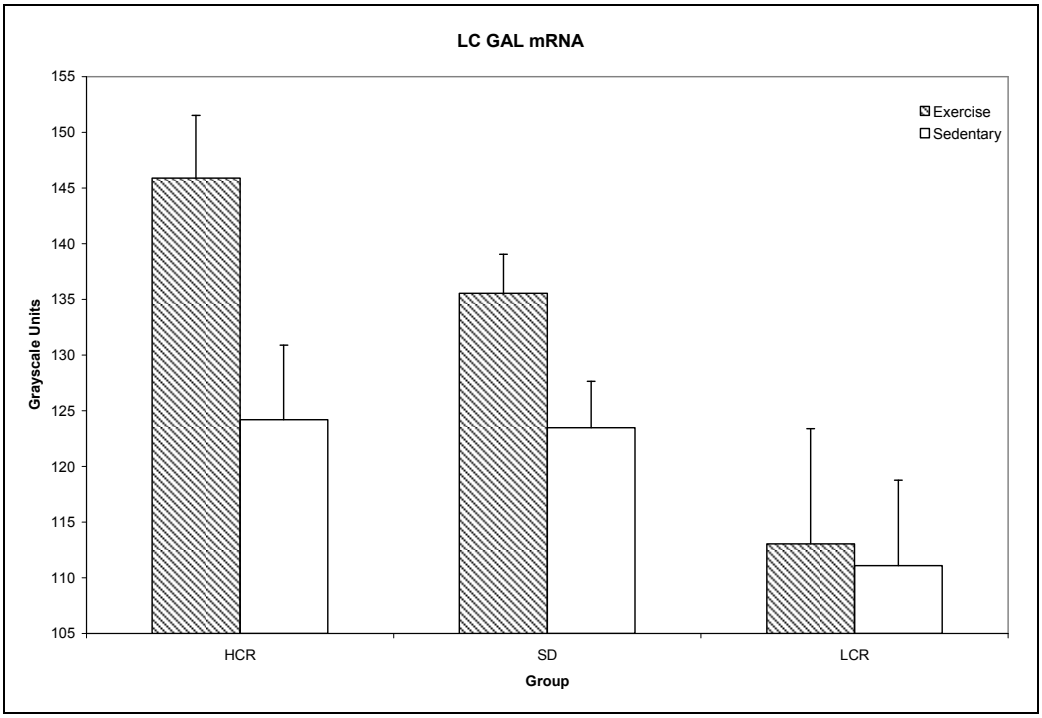

LC GAL mRNA
155
150
145
140
135
130
125
120
115
110
105
Grayscale Units
Exercise
Sedentary
HCR
SD
LCR
Group

Figure 4.5 Mean TH mRNA expression in the LC, grayscale units.

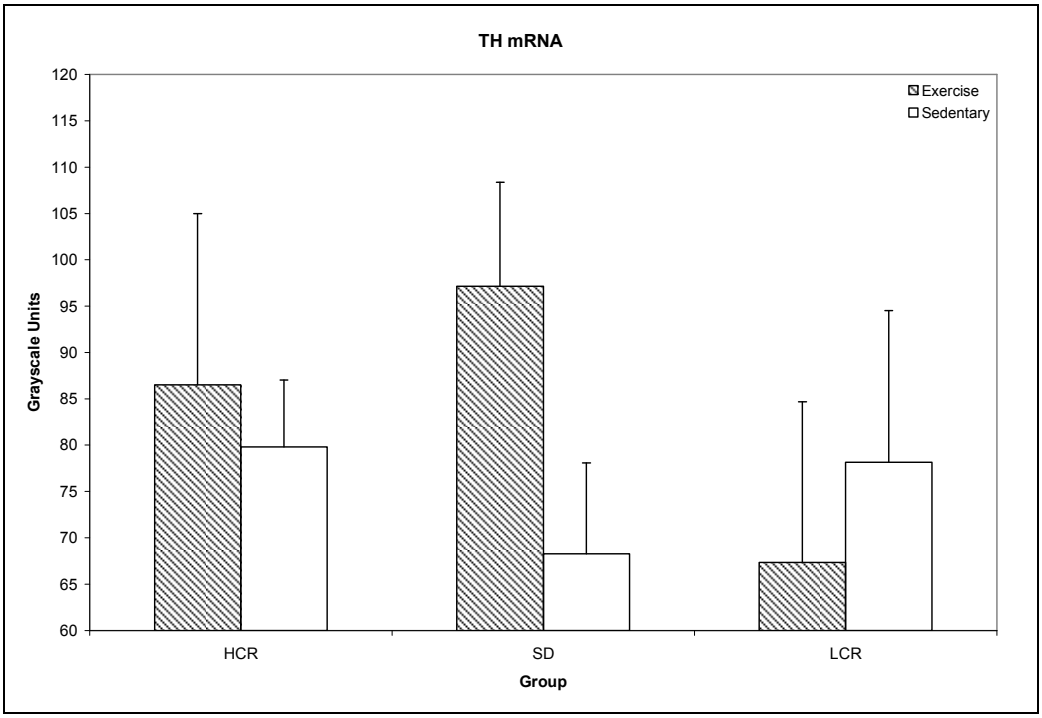
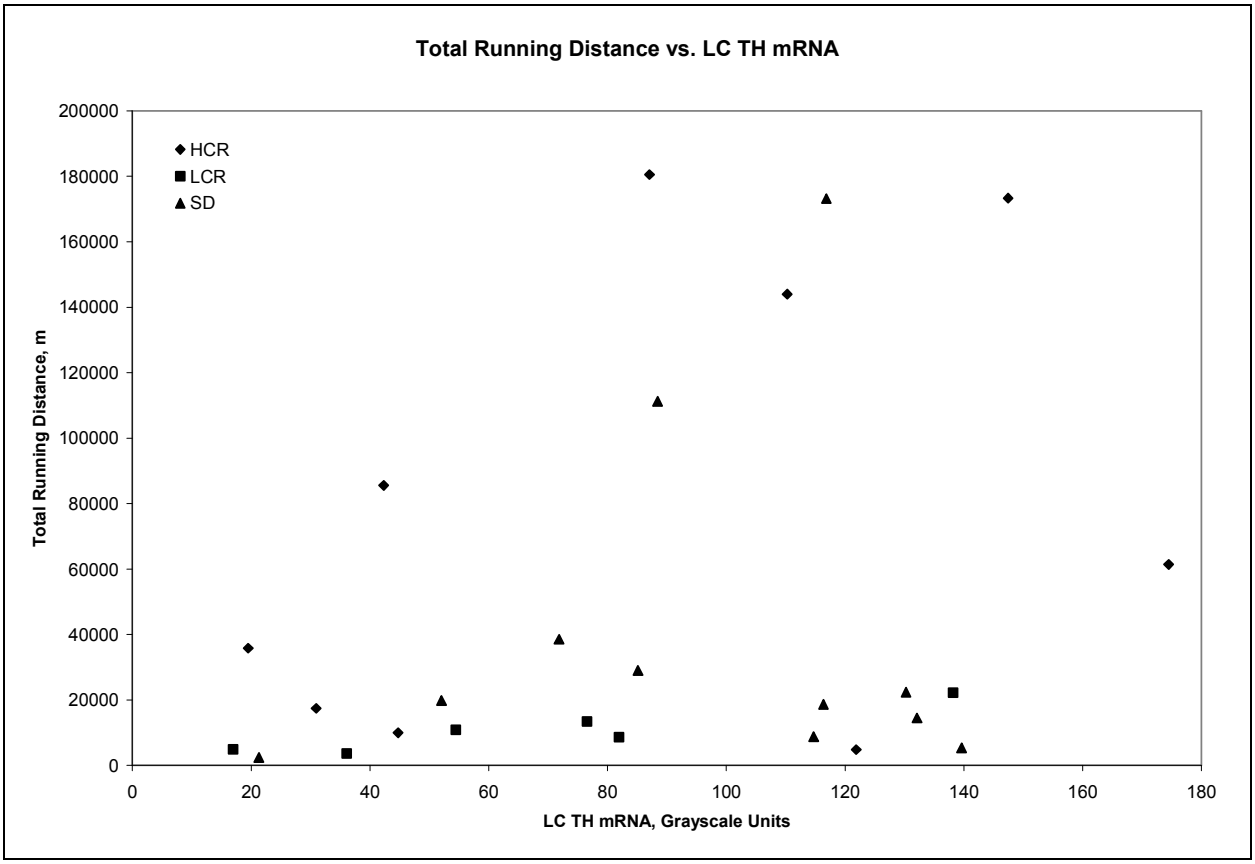

TH mRNA
Exercise
Sedentary
Grayscale Units
120
115
110
105
100
95
90
85
80
75
70
65
60
HCR
SD
LCR
Group

Figure 4.6 Total running distance (in meters) vs. TH mRNA (grayscale units).


Total Running Distance vs. LC TH mRNA
Total Running Distance, m
LC TH mRNA, Grayscale Units
HCR
LCR
SD
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0
20
40
60
80
100
120
140
160
180

CHAPTER 5

GENERAL DISCUSSION

The present studies examined the relationship between voluntary wheel running, seizure severity, and GAL activity in the LC. The anticonvulsant effect of exercise, whose foundation presumably lies at least in part in the activity-induced enhancement of GAL signaling, was examined by related means of measuring seizure severity; a modified Racine scale and SWD frequency via subdural EEG measurements. The second project yielded valuable information about the association between running distance and LC GAL expression, and that aerobic running capacity influences GAL expression as far as actual running distance is concerned. Voluntary wheel running did not induce a response in TH gene expression in the LC, further suggesting the minimal stress involved in the exercise protocol.

One of the main hypotheses examined, that voluntary wheel running influences seizure severity, was not statistically supported. However, sedentary rats did experience seizures at a level of severity the exercising rats did not reach. Since behaviorally the seizure severity was so apparently different between the groups to observers, and the seizure severity scale that was used was related to the SWD frequency in the EEG data, it is likely that exercise does influence seizure severity in some capacity. The enhancement of GALergic activity in the LC from running would produce effects in the hippocampus via dorsal noradrenergic bundle projections; in this way GAL modulates the spread of hyperexcitability in the particularly sensitive hippocampus.

The higher level of GAL mRNA LC expression in the HCR (and SD) rats compared to LCR rats demonstrated that running and GAL are related in a manner independent from internal adaptations to generations of selection for aerobic capacity. This relationship provides further evidence that simple activity wheel access produces GAL transcription effects regardless of ability to run without an associated NE effect. In another study of HCR and LCR rats, serotonin synthesis in the raphe was unaffected by treadmill running, although raphe 5-HT1B autoreceptor mRNA was higher in HCR rats (Foley, et al., 2006); importantly, these effect were observed with a 3 week latency between last treadmill run and in situ hybridization. An increase in striatal dopamine activity in wheel running HCR rats compared to LCR rats has been observed (Waters, et al., 2008), reflecting differences in locomotor behavior and possibly motivation, or 'want', to run (Berridge, Robinson, & Aldridge, 2009). These HCR and LCR rats provide a unique model to understand the exercise-induced increase in GAL.

Underlying the mechanism behind running-induced upregulation of LC GAL is a vast constellation of physiological changes. Considering the involvement of the LC in cardiovascular homeostasis, it is not surprising that blood pressure would participate in this mechanism. LC GAL mRNA is increased in spontaneously hypertensive rats compared to age-matched control Wistar-Kyoto rats when prehypertensive but not hypertensive (Kunkler, Wang, & Hwang, 1994). Acute bouts of treadmill running is associated with reduced mean arterial pressure in spontaneously hypertensive rats, after running (Overton, Joyner, & Tipton, 1988). At least 4 weeks of free access to running wheels increased atrial and ventricular weight and aortic compliance with no change in aortic response to NE in Wistar-Kyoto rats (Kingwell, Arnold, Jennings, & Dart, 1997), and eight weeks of access to wheels dampened increases in systolic and

diastolic pressure and load in spontaneously hypertensive rats (Collins, Rodenbaugh, & DiCarlo, 2000).

The PGL pathway provides excitatory amino acid input to the LC, at least in part via action at kainaite receptors in the LC (Ennis & Aston-Jones, 1988; Ennis, Aston-Jones, & Shiekhattar, 1992). Neurons in the caudal portion of the PGL demonstrate patterns of firing that correspond to respiration, some even specific to inspiration and expiration (Li, Zheng, & Xu, 1996), presumably due to input from the Kolliker-Fuse nucleus (Dick, Bellingham, & Richter, 1994). The PGL is essential to the LC effects on vascular homeostasis, and its lesion also eliminated response of LC to sciatic nerve stimulation and tail-pinch (Aston-Jones, et al., 1991). The PGL is also connected to inferior collicular nuclei and cochlear nucleus, indicating the integration of an auditory component as well (Andrezik, Chan-Palay, & Palay, 1981). Thus the PGL integrates an array of sensory information, somatic and environmental, for the LC.

The other major input to the LC originates in the PRH (Aston-Jones, et al., 1986). PRH neurons are involved in gaze-holding (Baker, Gresty, & Berthoz, 1976). In fact, the PRH neurons receive input regarding all types of horizontal eye movement as well as vestibular input, possibly indicating information about head acceleration (Fukushima & Kaneko, 1995); it is also via the vestibular nuclei that proprioceptive information regarding the neck is integrated to the LC (Gdowski & McCrea, 2000). PRH projections to the LC cause a uniform inhibition of LC discharge (Ennis & Aston-Jones, 1989), though lesions of the PRH lesions fail to eliminate GABA activity in the LC (Aston-Jones, Shipley, & Grzanna, 1995).

The LC receives input from other systems as well (Singewald & Philippu, 1998). Significant concentrations of 5-HT has been found in the LC (Palkovits, Brownstein, & Saavedra, 1974), carried along projections to the LC originating from the dorsal (Cedarbaum &

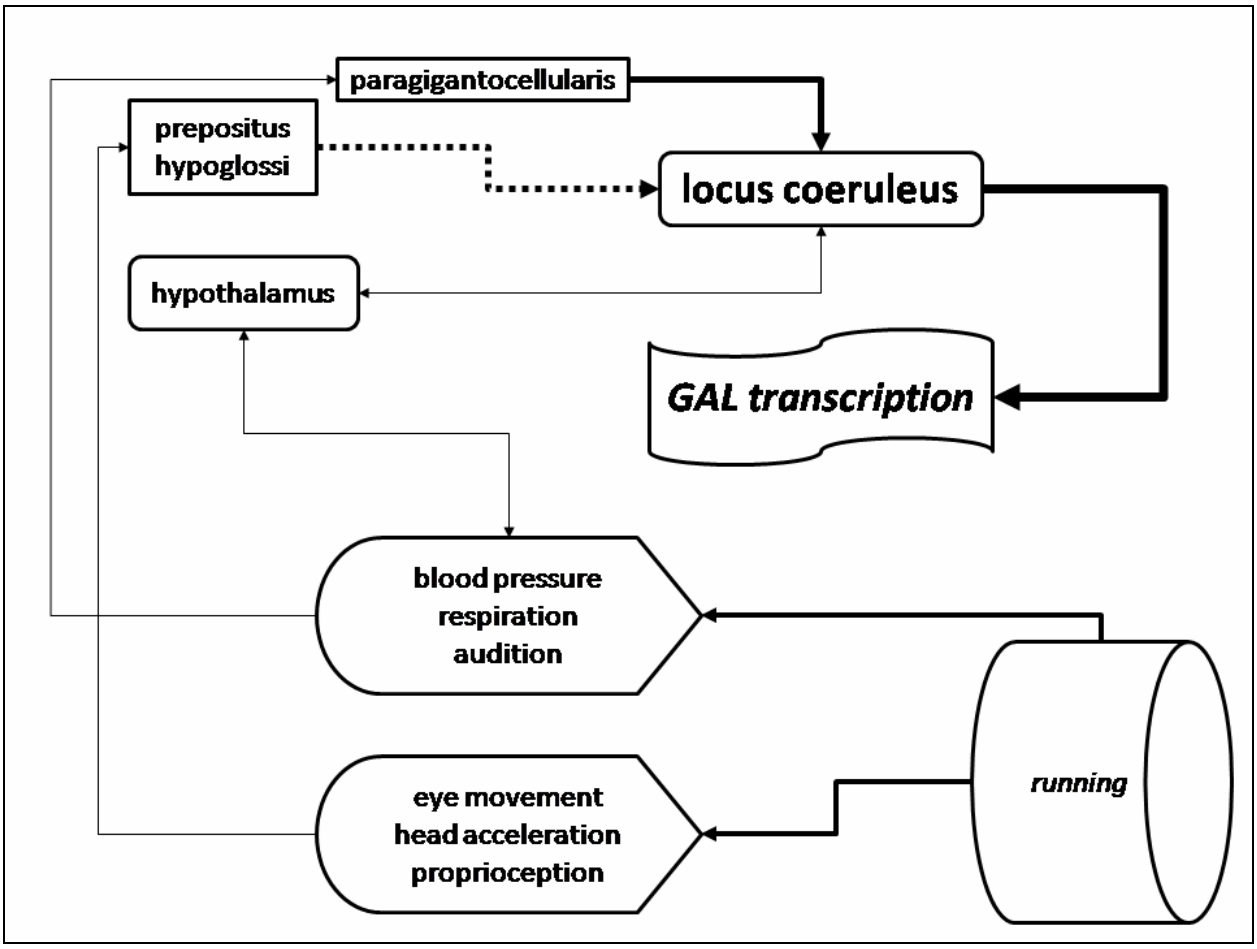
Aghajanian, 1978b) and caudal (Vertes & Kocsis, 1994) aspects of the raphe nuclei. The hypothalamic A13 cell group also provides dopaminergic input (Maeda et al., 1991). Barrington's nucleus provides a major source of CRF input and integrates abdominal visceral information to the LC (Rouzade-Dominguez, Curtis, & Valentino, 2001).

Importantly, the release of classical neurotransmitters and neuropeptides do not necessarily coincide; their differential release is dependent on pattern and frequency of activity (Hökfelt et al., 2000; Lundberg, 1996; Lundberg & Hokfelt, 1983). Comparably small elevations in cytoplasmic calcium can elicit neuropeptide release (Verhage et al., 1991), whereas amino acid secretion requires the higher calcium elevations provided by voltage-gated calcium channels near the active zone (Hökfelt, et al., 2000). These effects would account for the enhanced activity of GAL, but not TH, in the LC that is associated with running.

Conclusion

Many areas of the brain collaborate to provide extensive neuronal input to the LC, and the nature of each of the myriad inputs adapts to conditions of voluntary wheel running. This results in a complex modulation of input to the LC (figure 5.1), thereby affecting the LC firing rate in a convoluted manner. In this way the activity of the LC is modulated to release GAL without affecting TH activity. This prolonged modification to the activity of the LC may then result in a change to gene transcription for GAL while leaving alone other catecholamine activity in the LC.

Figure 5.1 Influence of wheel running on LC activity.


paragigantocellularis
prepositus hypoglossi
locus coeruleus
hypothalamus
GAL transcription
blood pressure
respiration
audition
eye movement
head acceleration
proprioception
running

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