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Activity-dependent and -independent control of circadian rhythms in mammalian skeletal muscle

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ABSTRACT

Autonomous biological rhythms allow organisms to coordinate internal processes with environmental conditions. Mammals exhibit a diverse array of both behavioral and physiological rhythms that are generated by an endogenous molecular timing system composed of a central pacemaker within the suprachiasmatic nucleus (SCN) of the hypothalamus in addition to autonomous oscillators within the cells of peripheral tissues. Previous reports have shown that clock-controlled outputs are essential for the temporally coordinated execution of many tissue-specific functions, yet specific entrainment pathways for skeletal muscle, a peripheral tissue that accounts for the majority of daily energy consumption, remain largely speculative. Studies suggest that both neural and humoral factors contribute to phase-coordinate the expression of rhythmic genes in peripheral tissues, and locomotor activity, autonomic innervation and metabolic signals resulting from food availability are all probable mediators of rhythmic gene expression in skeletal muscle. Here we investigated entrainment of the skeletal muscle core oscillator by selectively manipulating each proposed pathway *in vivo* while monitoring expression profiles of the core clock genes *Bmal1*, *Per1* and *Per2*. Monitoring circadian nucleocytoplasmic shuttling and transcriptional activity of the nerve activity-dependent sensor NFAT, we demonstrate that while some rhythmically expressed genes are strictly activity-dependent, motor innervation is not an important factor regulating phase entrainment of the core oscillator. Similarly, a chemical sympathectomy with 6-OHDA failed to significantly alter the phase of the core clock genes. However, two weeks of a restricted feeding schedule significantly shifted the phase of *Bmal1* expression in skeletal muscle as in liver, while, surprisingly, both *Per1* and *Per2* expression lost rhythmicity. These results clearly show that the circadian transcriptome in skeletal muscle is composed of both activity-dependent and –independent genes, and furthermore, that entrainment of the skeletal muscle circadian oscillator depends on metabolic factors rather than on neural activity.

SOMMARIO

I ritmi biologici autonomi permettono agli organismi di coordinare i processi interni con le condizioni ambientali. I mammiferi mostrano diversi tipi di ritmi comportamentali e fisiologici, che sono generati da un orologio molecolare endogeno composto da un "pacemaker" centrale presente all'interno del nucleo soprachiasmatico (SCN) dell'ipotalamo e degli oscillatori autonomi all'interno delle cellule dei tessuti periferici. Studi precedenti hanno indicato che i segnali generati da questo sono essenziali per la coordinazione temporale di molte funzioni tessuto-specifiche. Tuttavia, rimane in gran parte speculativo quale sia ruolo specifico di questo sistema nel muscolo scheletrico, un tessuto periferico in cui avviene la maggior parte del consumo di energia quotidiano. Alcuni studi suggeriscono che sia i fattori neuronali che quelli umorali contribuiscono all'espressione dei geni ritmici nei tessuti periferici e dall'attività locomotoria e che l'innervazione autonoma ed i segnali metabolici regolati dalla disponibilità di cibo sono i probabili mediatori dell'espressione di geni ritmici nel muscolo scheletrico. In questo lavoro di tesi abbiamo studiato il ruolo dell'orologio biologico nel muscolo scheletrico, analizzando selettivamente ogni via di segnale proposta "*in vivo*" e controllando i profili di espressione dei geni dell'orologio *Bmal1*, *Per1* e *Per2*. L'osservazione della traslocazione circadiana nucleo-citoplasma e l'attività trascrizionale del fattore NFAT, un sensore dell'attività nervo-dipendente, ci ha permesso di dimostrare che l'espressione dei geni dell'orologio e' direttamente correlato con l'attività e che l'innervazione non e' essenziale nella regolazione dell'orologio biologico. Similmente, l'uso di un composto chimico (6-hydroxydopamine) non ci ha permesso di alterare significativamente la fase dei geni dell'orologio. Tuttavia, sottoponendo gli animali a due settimane di programma d'alimentazione limitato abbiamo osservato un significativo spostamento di fase dell'espressione *Bmal1* nel muscolo scheletrico e nel fegato, mentre, l'espressione sia di *Per1* che di *Per2* ha perso la fase di ritmo. Questi risultati indicano chiaramente che la transcritoma circadiano nel muscolo scheletrico comprende sia geni attività-dipendente che indipendente e, che l'oscillazioni dell'orologio circadiano nel muscolo scheletrico dipendono dai fattori metabolici e non dall'attività neuronale.

INTRODUCTION

1. Circadian Rhythms

1.1 Circadian rhythms are an adaptive response

Biological rhythms are a consequence of life on earth. Stable light/dark cycles resulting from the earth's rotation about its axis set the stage for a great evolutionary pressure to play out, the result of which is the temporal organization of behavior and/or physiology in almost all species. Physiological timing is such a fundamental aspect of life that it can easily be taken for granted; however, it has been demonstrated in species as diverse as flies, bacteria and plants, that the more closely an organism coordinates its biological processes in anticipation of rhythmic variations in environmental conditions, the greater its reproductive fitness, and the greater the likelihood that its genes will make it to the next generation (Beaver *et al.*, 2002; Woelfle *et al.*, 2004; Dodd *et al.*, 2005). In other words, as the often cyclical nature of fashion and politics attests, those who fail to "keep up with the times" do so at their peril. The coordination of internal time in anticipation of external time is accomplished by an intricate system of molecular oscillators that organizes various timed physiological tasks such as daily variations in blood pressure and cell division, thus maximizing efficient operation of the whole organism.

1.2 Entrainment of rhythmic clock gene expression in the CNS and peripheral tissues

Circadian rhythms are generated by an endogenous timing system composed of a central pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus and autonomous molecular oscillators in the cells of peripheral tissues. The SCN receives direct synaptic input from the retina via the retinohypothalamic tract (Fig. 1), allowing timing information from the environmental light/dark cycle to directly synchronize the phase of the SCN core oscillator. Central entrainment of peripheral circadian oscillators has not been fully elucidated, but is thought to rely on the integration of various signaling pathways, including behavioral outputs such as activity-rest and feeding-fasting cycles, neuroendocrine signaling, and other outputs which are transmitted from the SCN to the periphery via humoral factors (McNamara *et al.*, 2001) and polysynaptic autonomic circuits (Ueyama *et al.*, 1999; Kalsbeek *et al.*, 2007).

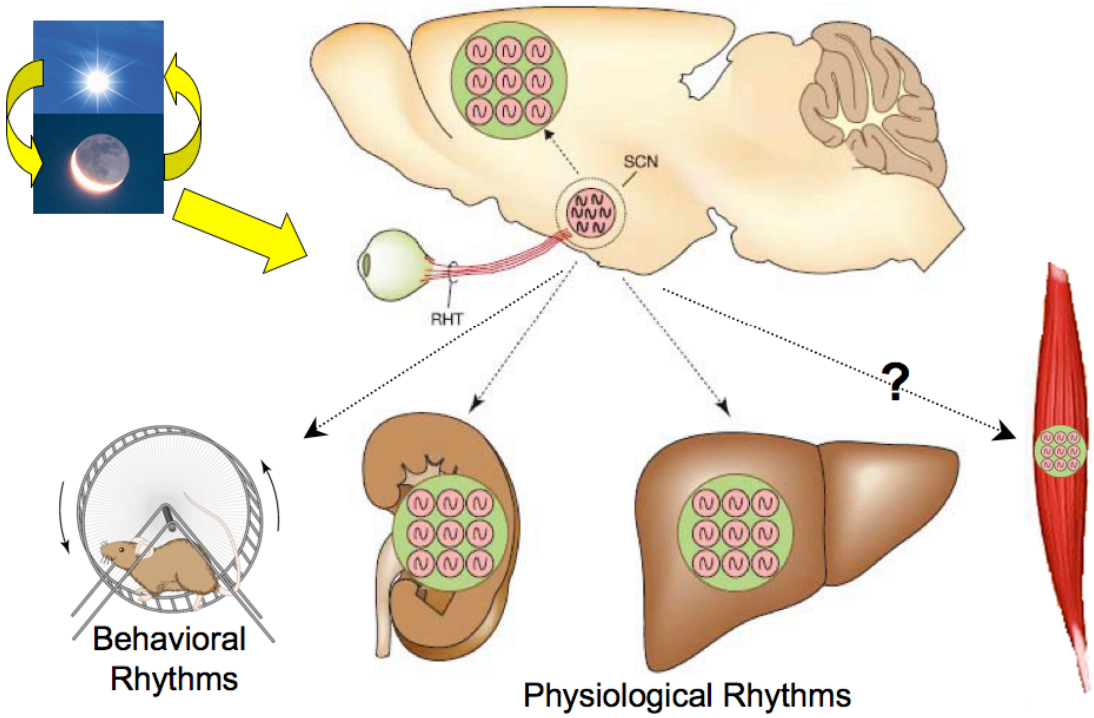


Figure 1. Timing information from the day/light cycle is directly transmitted to the SCN via the retinohypothalamic tract (RHT). Rhythmic SCN outputs include behavioral and physiological rhythms that are transmitted to peripheral tissues via neural signals and humoral factors. Entrainment signals for skeletal muscle circadian gene regulation are unknown, but may include locomotor activity rhythms, autonomic signaling and metabolic factors resulting from feeding time.

1.3 Rhythmic clock gene expression and transcriptional-translational feedback loops

At the cellular level (Fig. 2), the core of this rhythm-generating oscillator is composed of two interlocking regulatory feedback loops (Reppert & Weaver, 2002; Sato *et al.*, 2006; Takahashi *et al.*, 2008). In the first loop, the bHLH-PAS domain-containing transcription factors Clock and Bmal1 form heterodimers in the cytoplasm and translocate to the nucleus where they bind to E-box sequences, promoting transcription of *Per* and *Cry* genes. Through negative feedback, Per and Cry proteins form heterodimers in the cytoplasm and translocate to the nucleus, repressing the formation of the Bmal1/Clock complex. In a second regulatory loop, Bmal1/Clock activation leads to transcription of *Rev-erbα*, which, through its inhibition of the ROR enhancer element, inhibits *Bmal1* transcription (Preitner *et al.*, 2002). This core cellular oscillator regulates the rhythmic expression of physiologically relevant genes in a tissue-specific manner (Miller *et al.*, 2007), and most if not all cells of the body contain their own circadian clockwork (Panda *et al.*, 2002).

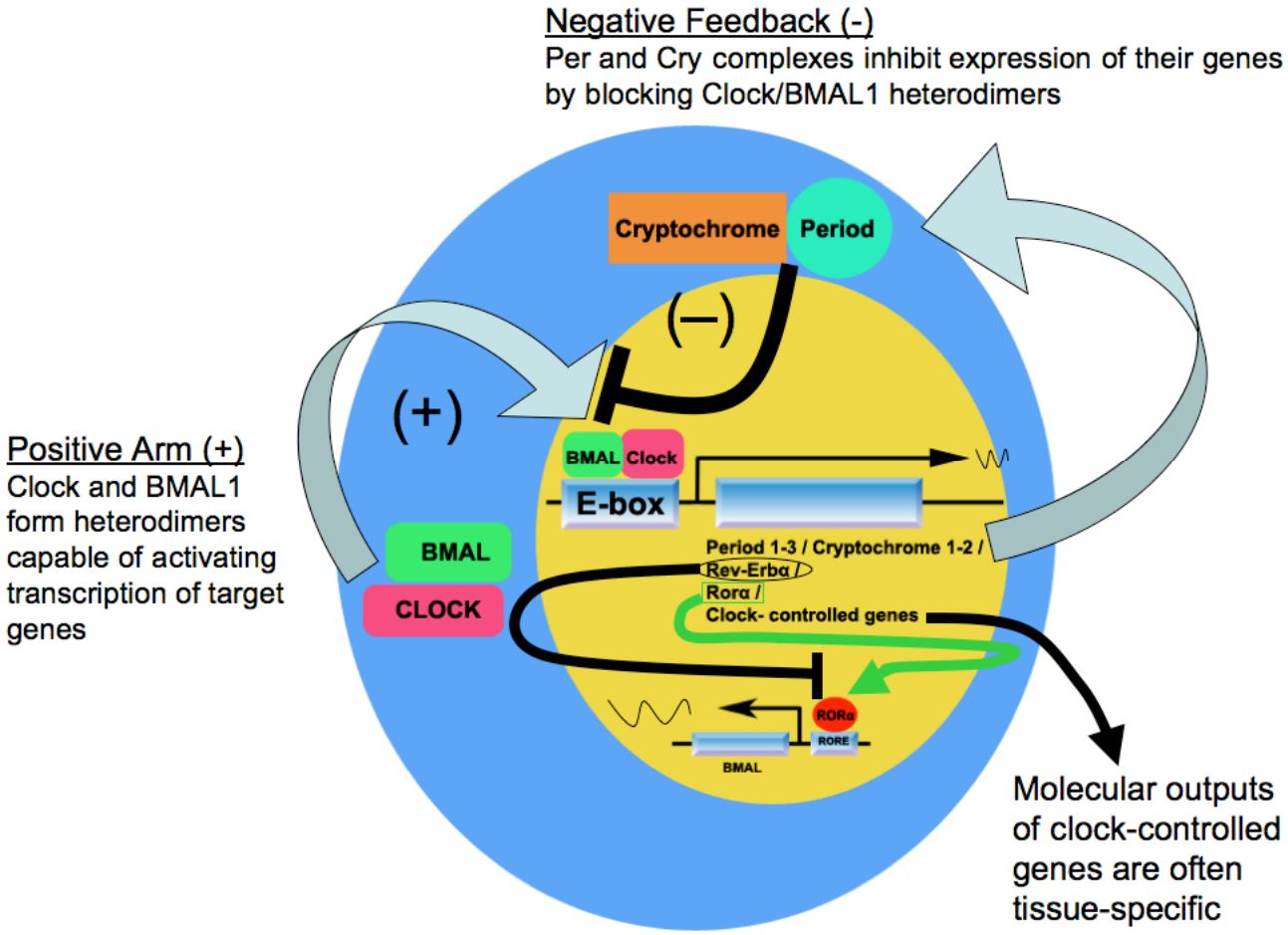


Figure 2. Circadian timing is a result of transcriptional/translational feedback loops. The positive arm of the clock depends on the protein products of the *Clock* and *Bmal1* genes, basic helix–loop–helix factors that form heterodimers capable of activating the transcription of the *Period* (*Per1-3*) and *Cryptochrome* (*Cry1-2*) genes. In negative feedback, the cyclic levels of PER and CRY proteins form complexes and accumulate in the nucleus where they inhibit expression of their genes by acting on CLOCK / BMAL1 heterodimers. The orphan nuclear receptors Rev-erbα and RORα form additional feedbacks that stabilize the clock rhythm by transcriptional regulation of *Bmal1*. Clock-controlled genes are the rhythmically expressed molecular outputs from the core clock mechanism, and are often tissue-specific, reflecting the physiological needs of a particular tissue or organ.

1.4 The importance of circadian timing in skeletal muscle physiology

Skeletal muscle occupies a uniquely central role both in metabolism and locomotor activity, making it an ideal focal point for circadian rhythms research both in health and disease. The importance of global circadian timing is particularly evident in shift workers, who exhibit an increased prevalence of metabolic syndrome, high BMI and cardiovascular problems including heart attacks and sudden death (Karlsson *et al.*, 2001; Ellingsen *et al.*, 2007), in addition to an increased risk for certain kinds of cancer (Moser *et al.*, 2006). Furthermore, studies have established that local timing in peripheral tissues is important for normal organ function. For example, a cardiomyocyte-specific circadian clock mutant showed altered myocardial contractile function and metabolism in addition to an altered gene expression profile (Bray *et al.*, 2008). Disruptions of circadian timing both globally and locally within skeletal muscle could be expected to give rise to similar consequences, and studies have already highlighted the importance of circadian timing in skeletal muscle physiology.

Rhythmic locomotor activity is completely disrupted in *Bmal1* single KO mice, *Per1* and *Per2* double KO mice, and *Cry1* and *Cry2* double KO mice (Bae *et al.*, 2006). More importantly, *Bmal1* KO mice also show age-related reductions in muscle mass, including decreases in fiber number and diameter (Kondratov *et al.*, 2006). These reductions are reminiscent of sarcopenia, the universal loss of muscle mass and strength that occurs in healthy ageing (Ryall *et al.*, 2007), suggesting that *Bmal1* may be involved in the age-related decline of muscle quantity and quality. Interestingly, muscle-specific rescue of *Bmal1* in *Bmal1-/-* mice was sufficient to restore body weight, total locomotor activity levels and longevity to wild-type levels, whereas brain-specific rescue only restored rhythmic locomotor activity, yet total activity levels remained significantly lower than muscle-specific *Bmal1*-rescued and wild-type mice (McDearmon *et al.*, 2006). Furthermore, a functional clock is important for normal muscle contractile function, as *Clock* mutant mice show decreases in force production (Miller *et al.*, 2007), *Per2* mutant mice show reduced muscle strength and endurance (Bae *et al.*, 2006; Albrecht *et al.*, 2007), and skeletal muscles of *Rev-erbAα*-deficient mice exhibit alterations in contractile protein content, showing a shift in myosin heavy chain composition (Downes *et al.*, 1995; Pircher *et al.*, 2005).

1.5 Entrainment signals for skeletal muscle remain speculative, and may include contributions from feeding cycles, locomotor activity rhythms and fluctuating sympathetic tone

Recent studies indicate that phase entrainment of peripheral oscillators probably relies on contributions from multiple sources (Balsalobre *et al.*, 2000b; Vujovic *et al.*, 2008) including neuronal pathways and humoral signals. Specific entrainment factors for many peripheral tissues, in addition to their effector signaling pathways, have recently begun to emerge (Stratmann & Schibler, 2006), yet, compared with more studied peripheral organs such as liver, data on the entrainment of skeletal muscle remains largely speculative and unexplored.

As in other peripheral tissues, skeletal muscle loses rhythmic clock gene expression following SCN lesions. However, unlike other peripheral tissues such as liver and kidney, transplantation of a functional SCN into SCN-lesioned Syrian hamsters fails to reinstate normal gene oscillation in skeletal muscle, despite restoring locomotor activity rhythms (Guo *et al.*, 2006). Similarly, parabiosis experiments in mice showed that non-neural signals, either behavioral or bloodborn, are adequate to restore circadian rhythms of clock gene expression in liver and kidney of SCN-ablated parabiosed partners, but not for heart, spleen or skeletal muscle, suggesting tissue-specific entrainment pathways (Guo *et al.*, 2005).

The investigation of entrainment signals for skeletal muscle is complicated by its dual roles in both locomotion and metabolism. Rhythmic clock gene expression in some peripheral tissues, including liver, heart, kidney and pancreas can be uncoupled from the SCN oscillator by restricting food availability (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). If nocturnal animals such as mice and rats are forced to eat during the light phase, when they are normally asleep, the circadian expression profiles of various clock genes in many peripheral tissues will shift phase by 12 hours, while clock gene expression in the SCN remains unaffected. The dominance of the feeding-fasting cycle as a skeletal muscle *Zeitgeber*, or entrainment signal, has not been established. However, well-noted physiological changes associated with restricted feeding regimens, including altered circadian profiles of plasma glucose, insulin, corticosterone, ketone bodies, and free fatty acids would also conceivably be expected to alter circadian gene expression in skeletal muscle.

Further complicating investigations into the entrainment of skeletal muscle by feeding is the associated occurrence of food anticipatory activity (FAA), an increase in locomotor activity in the hours immediately preceding a scheduled meal (Aschoff, 1991). This phenomenon was first observed in the 1920s by Curt P. Richter while working in the laboratory of the behaviorist John B. Watson

(Davidson, 2006). FAA has since been shown to be controlled by an endogenous circadian oscillator outside of the SCN, the so-called "food-entrainable oscillator" (FEO), as it is entrained by daily feeding schedules in SCN-lesioned animals (Stephan *et al.*, 1979). Similarly, corticosterone and body temperature rhythms appear to be under control of the FEO, as both can be re-entrained by scheduled meals in rats after SCN lesions (Krieger *et al.*, 1977). Any investigation of peripheral oscillator entrainment using restricted feeding regimens must take these issues into account when interpreting results.

Microarray studies of skeletal muscle have uncovered a large cluster of rhythmic genes coinciding with the middle of the activity/feeding phase (Miller *et al.*, 2007), and resistance exercise has been shown to modulate the expression of core oscillator genes, including *Bmal1* and *Per2* (Zambon *et al.*, 2003). These data suggest locomotor activity, including the increased contractile activity and/or associated changes in cellular metabolism, may be an important entrainment factor for clock gene expression in skeletal muscle (McCarthy *et al.*, 2007; Miller *et al.*, 2007).

Another possible entrainment signal is provided by daily fluctuations in sympathetic tone. While the sympathetic nervous system has been demonstrated to communicate timing information from the SCN to many other peripheral tissues for circadian gene regulation, its role in regulating circadian gene expression in skeletal muscle has not been investigated. Tract-tracing studies using Bartha's K strain of pseudorabies virus (PRV) have demonstrated direct polysynaptic circuits between the SCN and many peripheral tissues via sympathetic nervous system efferents (Bartness *et al.*, 2001). To date, liver, brown adipose tissue, thyroid gland, kidney, bladder, spleen, adrenal medulla, and the adrenal cortex have all been shown to be regulated by sympathetic innervation (Bartness *et al.*, 2001; Terazono *et al.*, 2003), and the list of tissues under sympathetic regulation continues to grow as additional studies are performed. Skeletal muscle also receives direct input from the sympathetic nervous system (Barker & Saito, 1981; Rotto-Percelay *et al.*, 1992), suggesting a possible role for sympathetic regulation of circadian gene expression in skeletal muscle.

Emerging evidence also suggests that metabolism is intimately linked with the circadian regulation of gene expression in skeletal muscle (Green *et al.*, 2008). Recent studies have identified several energy-sensing molecules that are able to transduce changes in cellular energy status into altered muscle gene programs and contribute to muscle plasticity (Freyssenet, 2007). Metabolic messengers, including AMP, O_2 levels, intracellular free fatty acids, and NAD^+ all signal variations in energy homeostasis to respective energy sensing molecules incuding AMP-activated protein kinase (AMPK), hypoxia-inducible factor-1 (HIF-1), peroxisome proliferator-activated receptors (PPARs),

and sirtuins (Sirt1, 3). Furthermore, transcriptome profiling has revealed many genes involved in metabolism that are rhythmically expressed in skeletal muscle (Yang *et al.*, 2006; McCarthy *et al.*, 2007), suggesting that the skeletal muscle clock plays an important role in nutrient processing and energy homeostasis (Asher *et al.*, 2008).

2. NFAT is a nerve activity-dependent sensor in skeletal muscle

In order to investigate possible activity-dependent regulation of circadian gene expression in skeletal muscle, we monitored translocation and transcriptional activity of NFAT, a nerve activity-dependent transcription factor.

2.1 The calcineurin-NFAT pathway in skeletal muscle

Nerve activity-dependent depolarization of the plasma membrane leads to the activation of the Ca^{2+}/Calmodulin-dependent serine/threonine phosphatase calcineurin (Cn). Activated Cn dephosphorylates the inactive, cytosolic forms of four members of the NFAT family of transcription factors (NFATc1-c4), causing their rapid nuclear accumulation and facilitating binding to target genes. This signaling pathway mediates the calcineurin-dependent regulation of skeletal muscle fiber type, particularly activity-dependent myosin switching (McCullagh *et al.*, 2004) and the induction and maintenance of the slow muscle gene program (Serrano *et al.*, 2001).

2.2 Nerve activity-dependent NFAT nucleocytoplasmic shuttling in skeletal muscle

NFAT nucleocytoplasmic shuttling (Fig. 3) is a key component of Cn-NFAT signaling in all relevant cell types and depends on the phosphorylation state of the SRR-1 and SP motifs, two serine-rich motifs that are conserved within the NFAT family. After dephosphorylation by Cn, NFAT translocates to the nucleus and activates or represses target genes, often with binding partners including other transcription factors or co-activators. Nuclear accumulation is a rapid event (Tothova *et al.*, 2006), reaching a maximum level within minutes of stimulation. On the other hand, NFAT nuclear export is a much slower event, lasting up to an hour (Tothova *et al.*, 2006), and depends on the activity of different kinases including CKI and GSK3 which respectively phosphorylate the SRR-1 and SP motifs. It is thought that the activity of these different export kinases contributes to signal specificity by regulating nuclear export kinetics.

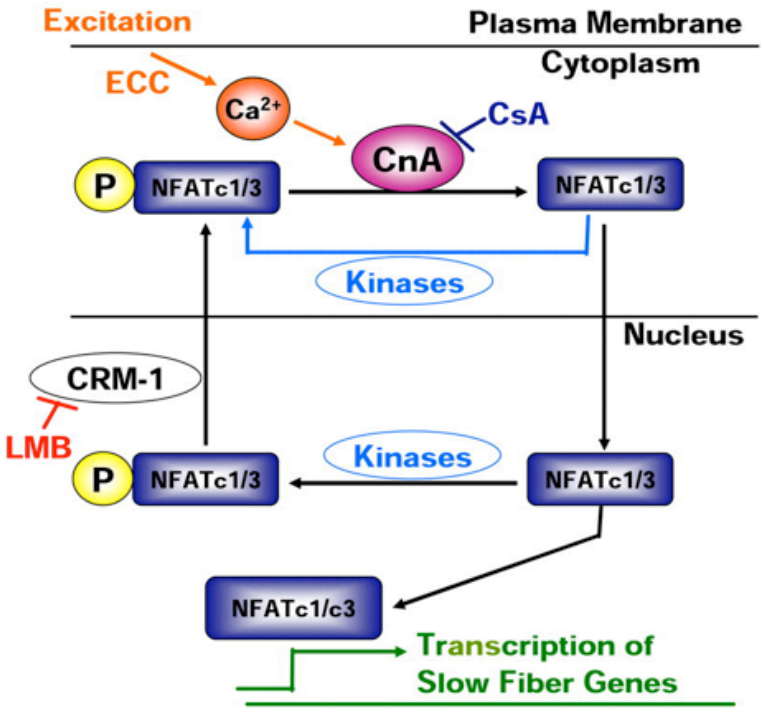


Figure 3. Nerve stimulation leads to an increase in intracellular Ca^{2+} and the activation of the phosphatase calcineurin (CnA), which is inhibited by Cyclosporin A (CsA). Activated CnA dephosphorylates the inactive cytoplasmic form of NFAT, leading to its rapid nuclear translocation. Once in the nucleus, NFAT binds to the promotor regions of target genes, initiating transcription, for example, of slow *MyHC I* which initiates the slow fiber gene program. Export kinases, including CKI and GSK3, phosphorylate nuclear NFAT, leading to its nuclear export via the nuclear export receptor chromosome region maintenance 1 (CRM-1), which is inhibited by leptomycin B (LMB). (adapted from Shen *et al.*, 2006)

NFAT nucleocytoplasmic localization also depends on the level of activation of the individual muscle fibers (Tothova *et al.*, 2006). It was previously shown that specifically NFATc1 is predominantly nuclear in slow tonic muscles like the soleus, and predominantly cytoplasmic in fast muscles like the tibialis anterior. The nucleocytoplasmic localization was shown to depend strictly on the level of activation, as electrical stimulation of a fast muscle with a slow tonic nerve pattern causes the nuclear accumulation of NFATc1, whereas in the absence of nerve input, whether by denervation or by anesthesia, NFATc1 exits the nucleus of slow muscles and resides predominantly in the cytoplasm. The specificity with which NFAT responds to different levels of nerve activity makes it an extremely sensitive and useful tool for monitoring and comparing activity levels of muscle fibers *in vivo*, particularly in muscles with different activity levels such as the fast tibialis anterior and the slow soleus, two muscles with very different activity levels and metabolic requirements.

2.3 Nerve activity-dependent NFAT transriptional activity in skeletal muscle

As mentioned above, NFAT mediates the Cn-dependent skeletal muscle fiber-type switching and maintenance of the slow gene program. This has been demonstrated and confirmed by a host of *in vitro* and *in vivo* studies. Chronic stimulation of cultured myotubes induces the expression of slow *MyHC I* (Kubis *et al.*, 2002). Similarly, mimicking this effect by stimulating the Cn pathway with the Ca^{2+} ionophore A23187 in cultured myotubes also increased expression of slow *MyHC I* (Meissner *et al.*, 2001; Kubis *et al.*, 2002). Cyclosporin A, a Cn inhibitor, prevented the up-regulation of slow *MyHC I* in both cases. Furthermore, single isolated skeletal muscle fibers from mouse flexor digitorum brevis, a muscle comprised primarily of fast-twitch fibers which do not express detectable levels of slow *MyHC I* mRNA, were stimulated with an impulse pattern mimicking a slow motor neuron firing pattern, and produced detectable levels of slow *MyHC I* transcripts (Liu & Schneider, 1998). These *in vitro* experiments have been confirmed using electrical stimulation of fast-twitch skeletal muscles in vivo (Kirschbaum *et al.*, 1989; Windisch *et al.*, 1998).

3. The sympathetic nervous system and skeletal muscle

3.1 The sympathetic nervous system is part of the autonomic nervous system

The autonomic nervous system is a major component of the peripheral nervous system, and is customarily distinguished from the somatic nervous system on the basis of voluntary control. This distinction may be considered somewhat artificial in that some autonomic functions, such as breathing, can also function under conscious control. Furthermore, both the somatic nervous system and the autonomic nervous system have several basic features in common. Namely, they share a similar embryological origin and are made up of similar neurons, they are difficult to separate anatomically, and, to a large extent, their activities depend upon each other (Pick, 1970). However, both structural and functional evidence points to the usefulness of such a distinction. These arguments aside, it is agreed that the autonomic nervous system largely regulates unconscious processes that maintain homeostasis, and its effects are often regulatory or modulatory in nature.

Autonomic neurons are classified as either efferent (motor) or afferent (sensory), and, in further contrast to somatic motor nerve fibers, which travel uninterrupted from the central nervous system to striated muscles, efferent autonomic pathways consist of a series of two neurons: a preganglionic neuron which has its cell of origin in the central nervous system and its axon in the periphery, and a postganglionic neuron with nerve fibers terminating in the effector organ. Preganglionic neurons usually possess a thin myelin sheath, whereas postganglionic nerve fibers are, as a rule, unmyelinated (or sometimes only very thinly myelinated). Furthermore, preganglionic neurons are strictly cholinergic, meaning they manufacture and release the neurotransmitter acetylcholine, whereas, depending on their target, postganglionic neurons are either cholinergic or adrenergic, meaning they manufacture and release the catecholamines adrenaline (epinephrine) or noradrenaline (norepinephrine).

A further distinction between the autonomic and somatic nervous systems is evident in the way that nerve fibers terminate at effector cells. Rather than the distinctive motor endplates and close neuromuscular junctions present in skeletal muscle, autonomic nerve fibers pass in the form of either one or several axons, shrouded by a more or less complete Schwann cell envelope. At variable intervals the terminals possess swellings containing aggregates of vesicles and granules of variable number and appearance. Accordingly, autonomic nerve terminals are referred to as *boutons en passant,* or *endings in passage*, and, while lacking direct synaptic contact with their targets, are able to evoke responses

over a relatively long distance (sometimes greater than 1μm), releasing neurotransmitter from free endings into a large extraneuronal space with no postjunctional specializations (Elenkov *et al.*, 2000). While classical synaptic transmission is relatively short and phasic, this “nonsynaptic” transmission is slow and tonic.

The autonomic nervous system is further divided functionally into complementary sympathetic and parasympathetic components. The sympathetic nervous system is responsible for Cannon’s famous "fight of flight" response, causing general arousal and energy generation while inhibiting digestion, whereas the parasympathetic nervous system promotes a "rest and digest" response, causing a return to resting function and enhancing digestion. In the sympathetic nervous system, nerves originate in the spinal cord and have a short preganglionic fiber and a long postganglionic fiber. Most ganglia form a paravertebral chain adjacent to the spinal cord, but a few prevertebral ganglia (the celiac, splanchnic and mesenteric ganglia) are located more distally to the spinal cord.

The sympathetic nervous system tends to discharge as a unit, producing diffuse activation of targets, whereas the parasympathetic system, in contrast, is capable of discreetly activating specific targets. Preganglionic sympathetic neurons synapse with a large number of postganglionic neurons, contributing to this widespread activation of target organs during increased sympathetic drive. At target sites, sympathetic postganglionic neurons release norepinephrine that binds to adrenergic receptors on the surface of cells in effector tissue. In addition, the release of epinephrine and norepinephrine from the adrenal medulla into the circulation enables the activation of target tissues throughout the body, including some that are not directly innervated by sympathetic nerves.

3.2 Catecholamine biosynthesis

Catecholamines are synthesized from tyrosine in noradrenergic nerve terminals (Fig. 4). Tyrosine is first converted to dihydroxyphenylanine (DOPA), the rate-limiting step in catecholamine synthesis, by tyrosine hydroxylase (TH) and finally to dopamine (DA). A carrier that can be blocked by reserpine transports dopamine into the vesicle, where it is converted to norepinephrine (NE) by dopamine-β-hydroxylase (DβH). In the adrenal medulla, NE is further converted to epinephrine. TH- and DβH-immunostaining are commonly used as specific markers to demonstrate noradrenergic innervation of various organs.

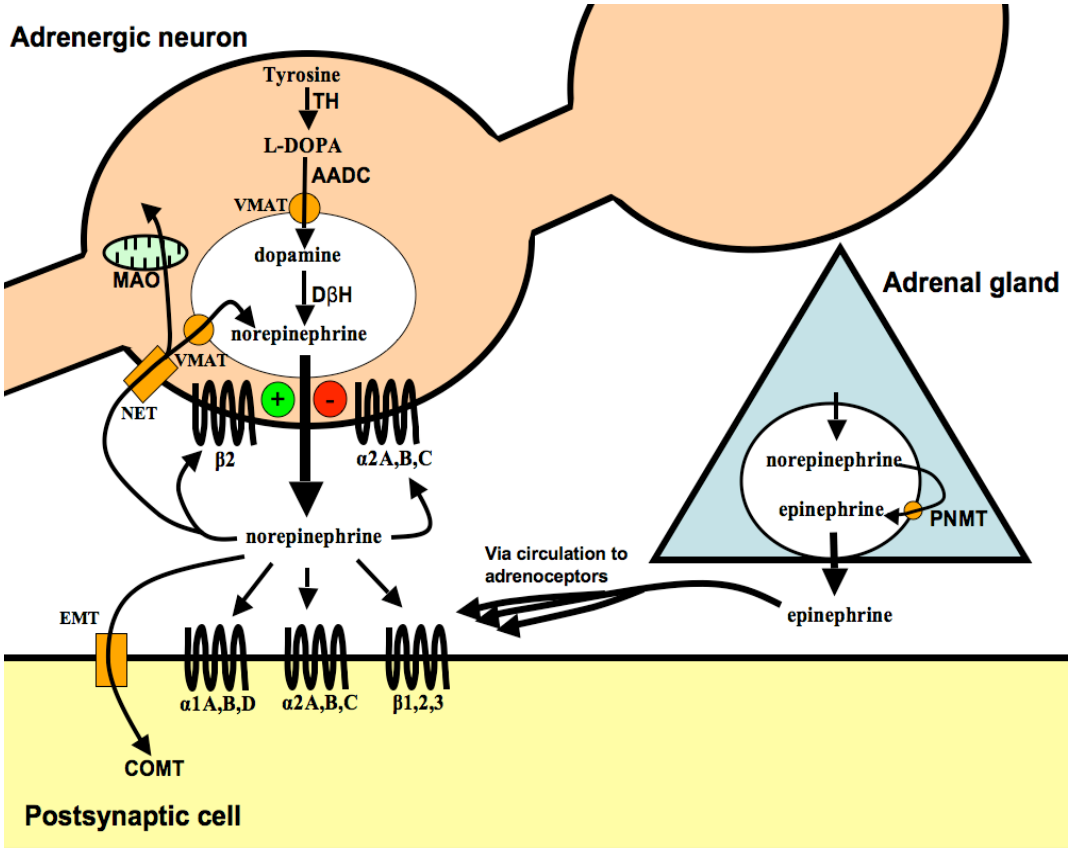


Figure 4. The adrenergic nervous system. Endogenous catecholamines are synthesized from tyrosine in sequential steps to L-DOPA by tyrosine hydroxylase (TH), dopamine by aromatic amino acid decarboxylase (AADC), and norepinephrine by dopamine β-hydroxylase (DβH). Phenylethanolamine N-methyltransferase (PNMT) synthesizes epinephrine within adrenal chromaffin cells. After exocytosis, norepinephrine may be transported back into synaptic vessicles by the norepinephrine transporter (NET) and vesicular monoamine transporter (VMAT) or into neighboring cells by the extracellular monoamine transporter (EMT). Catecholamines are metabolized by monoamine oxidase (MAO) or catechol O-methyltransferase (COMT). Binding of norepinephrine to α2-adrenoceptors on the surface of sympathetic terminals causes presynaptic feedback inhibition of norepinephrine release. On the other hand, binding of norepinephrine to β2-adrenoceptors on the surface of sympathetic terminals has been shown in some mouse heart and spleen to enhance norepinephrine exocytosis. (adapted from Hein, 2006)

3.3 Adrenergic receptors

The biological effects of epinephrine and norepinephrine are mediated by adrenergic receptors on the surface of target tissue cells. Historically, adrenergic receptor subtypes were classified according to the particular response elicited after the administration of various sympathetic stimulating drugs: those that elicited an excitatory response were designated alpha (α), and those that elicited an inhibitory response were designated beta (β). Subsequent investigations uncovered that the excitatory or inhibitory responses were dependent on the identity of associated G protein subunits rather than the identity of the adrenergic receptors, rendering this α/β distinction obsolete. However, this distinction remains popular and is still in use.

To date at least nine subtypes of adrenoceptors have been cloned, including six α- (α1A, α1B, α1D, α2A, α2B and α2C) and three β-subtypes (β1, β2 and β3), which have been found in different proportions in numerous tissues throughout the body. The physiological relevance of adrenergic receptor sybtype diversity has been investigated using both pharmacological ligands and the targeted deletion of receptor genes in mice (Philipp & Hein, 2004). The β-adrenoceptor family is the primary receptor subtype found in skeletal muscle, and particularly the β2 subtype (Lynch & Ryall, 2008). However, a smaller population of α-adrenoceptors are also present, often associated with blood vessels and muscle spindle fibers (Bombardi *et al.*, 2006), and are expressed in higher proportions in highly vascularized slow-twitch muscles (Rattigan *et al.*, 1986).

As in other tissues, skeletal muscle adrenoceptors are activated either by direct innervation or by circulating catecholamines, and are responsible for mediating a diverse array of physiological effects. For example, skeletal muscle blood flow is predominantly under direct autonomic neural control via sympathetic innervation, with sympathetic vasoconstriction regulated via both α1- and α2-adrenoceptors. The relative contributions of each receptor to sympathetic vasoconstrictor responses in skeletal muscle have yet to be defined, as do the signaling pathways through which they exert their respective effects (Thomas & Segal, 2004). However, α1- and α2-adrenoceptors show regional differences in distribution along the resistance network, with α1-adrenoceptors usually located on smooth muscle cells within larger resistance arterioles, and α2-adrenoceptors usually within smaller precapillary arterioles. Emerging evidence suggests that these regional differences in adrenoceptor distribution are behind the graded differences observed along the vascular bed in response to increased sympathetic drive. Particularly during exercise, sustained increases in sympathetic outflow leads to

regional differences in skeletal muscle blood flow, with proximal arterioles and feed arteries maintaining vasoconstriction, whereas distal arterioles exhibit a secondary metaboreceptor-induced relaxation.

As mentioned above, skeletal muscle contains a significant proportion of β-adrenoceptors that are mostly of the β2-subtype. However, about 7–10% are of the β1-subtype. Slow-twitch muscles, such as the soleus muscle, also have a greater density of β-adrenoceptors than fast-twitch muscles, such as the extensor digitorum longus (EDL). Although the functional significance of this difference in β-adrenoceptor density is not yet fully understood, the response to β-agonist administration appears to be greater in fast- than in slow-twitch skeletal muscles (Ryall *et al.*, 2002; Ryall *et al.*, 2006).

All adrenoceptors are members of the guanine nucleotide-binding G protein-coupled receptor (GPCR) family. Thanks to the large number of associated G protein subunits and their various combinations, numerous downstream effector pathways mediate the wide range of important physiological effects of adrenergic signaling in skeletal muscle, including adenylyl cyclase (AC)/cAMP-dependent secondary messenger pathways including protein kinase A (PKA)/CREB and Epac/Rap1/PI3K-Akt signaling (Lynch & Ryall, 2008).

3.4 In addition to perivascular innervation, skeletal muscle fibers and spindles receive direct sympathetic input

Historically, there has been some debate about whether skeletal muscle fibers receive direct input from the sympathetic nervous system, or whether perivascular sympathetic nerve terminals alone supply the non-circulating tissue catecholamines. Barker and Saito made a good summary of this debate:

> *"It is widely held that skeletal muscle fibers are not innervated by sympathetic axons. Claims that each muscle fibre is supplied with a sympathetic 'accessory ending' terminating near or within the somatic motor endplate (Boeke 1911, 1927), or that sympathetic axons exclusively innervate a group of muscle fibres responsible for 'plastic tone' (Hunter 1925), were refuted by Hinsey (1927), Wilkinson (1929), and Tower (1931). In studies with the light microscope Hinsey and Tower examined normal muscle and muscle deprived of its somatic innervation by degenerative spinal-root section and concluded that the sympathetic axons were entirely confined to blood vessels. On the basis of this evidence (reviewed by Hinsey 1934) it is assumed (Bowman & Nott 1969) that the effects of sympathetic stimulation on skeletal muscle contractions (other than those secondary to vascular changes) are due to noradrenaline diffusing to the muscle fibres from perivascular sympathetic varicosities. The only recent indication that these effects might, on the contrary, be mediated by direct innervation is contained in an*

ultrastructural study of thin non-myelinated axons in normal muscles by Santini & Ibata (1971). They made the incidental observation that such axons, considered by them to be sympathetic, were distributed apart from blood vessels among extrafusal muscle fibres."

Examining cat hindlimb muscles, Barker and Saito demonstrated that in addition to blood vessel varicosities, sympathetic axons terminate among extrafusal muscle fibers and some muscle spindles (Barker & Saito, 1981). Very few studies have investigated the physiological relevance of this particular anatomical arrangement.

3.5 Predominant role of muscle sympathetic nerve activity is to regulate blood flow in working muscles

Muscle sympathetic nerve activity (MSNA) can be directly measured by microneurography using fine metal electrodes inserted into the nerve fascicles of awake subjects. Because postganglionic sympathetic nerve fibers are unmyelinated and thus have a relatively slow conduction velocity, sympathetic discharges are easily distinguished (Grassi & Esler, 1999). These burst discharges are synchronized with the heartbeat and have been shown to regulate blood pressure, particularly in response to exercise. Furthermore, the magnitude of sympathetic outflow to skeletal muscle has been shown to depend on a host of factors, including intensity and duration of exercise, and the size of the contracting muscle mass (Thomas & Segal, 2004).

In humans, skeletal muscle accounts for about 45% of body mass and its blood distribution accounts for about 20% of cardiac output in resting conditions. However, during strenuous exercise, blood flow to active muscles can account for 90% of cardiac output. This shift in blood flow greatly affects systemic blood pressure, causing a compensatory increase in sympathetic outflow and vasoconstriction in skeletal muscle leading to a compensatory rise in systemic blood pressure termed the exercise pressor reflex (Smith *et al.*, 2006). However, as exercise progresses, a rise in exercise-induced metabolites, including nitric oxide (NO) and hypoxia-induced factors (Thomas & Segal, 2004), initiates vascular relaxation, thus increasing blood flow to working muscles. Conversely, prolonged exercise also causes skeletal muscle mechanosensors and metabosensors to further increase MSNA, which again leads to vasoconstriction in both active and nonactive vascular beds and a compensatory elevation in blood pressure. These compensatory vasoconstriction and vasodilation effects are carefully balanced to ensure that the nutritional demands of working muscles are met while protecting against

excessive elevations in capillary pressure and the resulting plasma fluid loss into the extravascular space of working muscles (Maspers *et al.*, 1991).

3.6 Sympathetic effects on muscle morphology

Pharmacological investigations paved the way for basic research of the sympathetic nervous system, and the majority of evidence demonstrating how skeletal muscle responds to sympathetic stimulation has been provided by studies using sympathomimetic drugs. β-adrenoceptor agonists (β-agonists) such as clenbuterol, a β2-agonist, were originally used as bronchiodialators to treat asthma. However, their use was also observed to significantly increase skeletal muscle mass while decreasing body fat, the so-called "repartitioning effect" (Lynch & Ryall, 2008). In addition to their therapeutic potential for treating muscle wasting disorders, these anabolic effects have also been harnessed in the livestock industry and by athletes.

The hypertrophy caused by β2-agonists like clenbuterol preferentially affects fast-twitch fibers and predominantly fast muscles like the EDL more than slow-twitch fibers or predominantly slow muscles like the soleus. This hypertrophy was observed to be functional (Zeman *et al.*, 1988), in that as muscle cross-sectional area increased, parallel increases in tetanic tension and force development rates were observed. There was also a shift in fiber-type composition of the muscles, with the ratio of fast- to slow-twitch fibers increased both in the fast EDL and the slow soleus. Similarly, the chronic blockade of adrenoceptors with the β2-antagonist butoxamine led to a ubiquitous reduction in glycolytic fast-twitch type II fibers and a reciprocal gain in type I fibers.

In contrast to the relatively ordered picture emerging from pharmacological studies on skeletal muscle contractile properties, data based on surgical sympathetic denervation are sparse, inconsistent (Kardos *et al.*, 2000), and often problematic. For the limited number of studies performed, a large variety of protocols have been used. Unfortunately, these often include different time scales, different animal models, and different muscles, making comparisons difficult. For example, 14 weeks after performing unilateral lumbar sympathectomy on female beagle dogs, Karlsson and Smith found a decrease in slow-twitch fibers and an increase in fast-twitch fibers and cross-sectional area of gracilis muscle (Karlsson & Smith, 1983), a multiply-innervated, predominantly fast-twitch muscle. Conversely, 12 weeks after unilateral lumbar sympathectomy, Henriksson and colleagues found no significant changes in either histochemical or enzymatic properties in male rat gastrocnemius (Henriksson *et al.*, 1985), a muscle of mixed fiber composition. While surprising, these descrepencies

may simply be due to a unique architectural feature of rat paravertebral sympathetic ganglia. In a rigorous anatomical study of the lumbar sympathetic nervous system in the rat, Baron and colleagues showed that in a majority of cases, ipsilateral segmental ganglia were either fused or connected with a small "bridge" of neuronal tissue, particularly in the L3 region innervating the gastrocnemius (Baron *et al.*, 1988). Furthermore, after unilateral application of HRP for tract tracing purposes, some labelling of the contralateral sympathetic chain was observed. This crossing of sympathetic neurons to contralateral ganglia could potentially complicate results in studies performing only unilateral sympathectomy and suggest that any study based on surgical sympathectomy would be well-complimented by parallel studies using pharmacological agents.

More recently, unilateral ablation of the cervical sympathetic chain in rabbits of undeclared sex led to varying metabolic and morphological alterations in masseter muscles which became more pronounced with time (Pai-Silva *et al.*, 2001). After an initially significant increase in type I fibers 3 months after sympathectomy, there was a progressive shift to a significantly higher frequency of type IIa and IIb fibers after 12 and 18 months. Interpretation of these results is difficult for several reasons. Besides failing to mention the sex of the rabbits used in the study, the choice of the rabbit masseter muscle is particularly problematic, since, besides exhibiting testosterone-driven sexual dimorphisms in muscle fiber-type composition, rabbit masseter muscle also shows a drastic shift in fiber composition from slow I to fast IIa occurring between 2-6 months of age (English & Schwartz, 2002). Again, consistency in experimental design is a necessary condition for useful comparisons of these denervation studies.

Aim of the project

Since skeletal muscle is essential both for metabolism and locomotor activity, we wanted to dissect the relative importance of each on rhythmic gene expression in skeletal muscle, focusing on the phase entrainment of the core oscillator, and on the transcription factors NFATc1 and –c3, which are known to act as activity sensors in skeletal muscle (McCullagh *et al.*, 2004).

MATERIALS AND METHODS

1. Animals

All experiments were performed on 2-5 month old adult male CD1 mice except for endogenous NFATc1 analyses, in which adult female Sprague Dawley rats were used due to the lack of a suitable antibody for mouse tissue. Experimental protocols were reviewed and approved by the local Animal Care Committee, University of Padova. Prior to all experiments, animals were first acclimated for 3 weeks to an isolated sound and temperature controlled room (22°C) under a 12hr light-dark regimen [lights on ZT0(6am), lights off ZT12(6pm)], with standard chow and water provided *ad libitum*. All tissues were collected immediately after cervical dislocation, snap frozen in isopentane cooled in N_2 liquid and stored at -80°C until subsequent use.

2. *In vivo* transfection

Transfection of plasmid DNA was performed as previously described (Tothova *et al*., 2006). Prior to surgery, mice were anesthetized by i.p. injection of a mixture of Zoletil 100® (a combination of Zolazapam and Tiletamine, 1:1, 10mg/kg, Laboratoire Virbac) and Rompun® (Xilazine 2%, 0.06ml/kg, Bayer). The soleus (SOL) and tibialis anterior (TA) muscles were surgically exposed and isolated by a small hindlimb incision. For monitoring NFAT nucleocytoplasmic shuttling, 25μg of a plasmid DNA mix (20μg of either NFATc1-GFP or NFATc3-GFP + 5μg H2B-RFP) was injected into each muscle. For NFAT reporter activity, 20μg of a plasmid DNA mix (15μg IL-4-NFAT + 5μg Rn-TK) was injected into each muscle. Plasmid injection was followed by electroporation with stainless steel spatula electrodes connected to a ECM830 BTX porator (Genetronics, San Diego, CA) with the following settings: 5 pulses of 20ms each with 200ms intervals. Voltage was adjusted according to the thickness of the muscle (5V/mm).

3. Plasmids

The construct for NFATc1-GFP was a gift from Rhonda Bassel-Duby, and consists of a full-length wild-type NFATc1 linked to EGFP (Chin *et al.*, 1998). In the NFATc3-GFP construct, a gift from Shoichiro Miyatake, the carboxyl-terminal domains of wild-type or truncated NFATx1 constructs were replaced with EGFP (Amasaki *et al.*, 2002). The Histone2B-RFP construct consists of histone2B linked to RFP and was a gift from Manuela Zaccolo. NFAT-dependent transcriptional activity was

monitored as previously described (McCullagh *et al*., 2004) using an IL-4-NFAT reporter construct consisting of 9 tandem NFAT-binding sites from the *interleukin* 4 gene fused to a basal *α-MHC* promoter linked to firefly luciferase (Braz *et al*., 2003). IL-4-NFAT was coinjected with Renilla-TK to normalize for transfection efficiency.

4. Short- and Long-Term Motor Denervation

Motor denervation was accomplished by removing a 1-2mm segment of the sciatic nerve high in the thigh of the anesthetized animal. The contralateral leg was left intact and used as a control. Muscles were collected at ZT 0 and 12, either 12 hours (short-term) or 7 days (long-term) after sciatic transection.

5. Restricted Feeding Schedule

For the restricted feeding group, access to food was restricted to the light phase (ZT0-12, 6am-6pm) for 14 days with bedding changed each night at lights off for both restricted and the *ad libitum* feeding groups in order to prevent the restricted feeding group from scavenging for food sources during the dark phase. Muscles were collected at ZT 0, 4, 8, 12, 16, and 20.

6. Chemical sympathectomy with 6-OHDA

To abolish sympathetic nerve activity in skeletal muscle, adult mice were treated with 6-OHDA (6-Hydroxydopamine hydrobromide, 200mg/kg, Sigma). Drug was prepared fresh for each treatment. 6-OHDA was dissolved in vehicle (0.1% ascorbate in saline) and injected i.p. on days 1, 3 and 6. Control animals received i.p. injections of vehicle. Muscles were collected on day 14 at ZT 0 and 12. This longer-term treatment protocol (2weeks) was selected to avoid noted side effects which could potentially complicate the interpretation of results, including significantly reduced food intake and alterations in locomotor activity (Terazono *et al.*, 2003) resulting from a short-term treatment (1week). In preliminary experiments testing the successful maintenance of the chemical sympathectomy in skeletal muscle over a longer time we observed that animals rapidly lost weight within the first two days of treatment. However, waiting two weeks after the start of 6-OHDA treatment before collecting the tissue, including a maintenance injection at day 6, is sufficient to restore body weight to control levels while maintaining a complete lack of sympathetic innervation as revealed by tyrosine hydroxylase IHC.

7. Detection and quantification of transfected and endogenous NFAT

Transverse cryosections (10µm) of muscles were used in all analyses. NFATc3-GFP labeling was enhanced with polyclonal rabbit anti-GFP (1:200, Molecular Probes) and Cy2-conjugated anti-rabbit IgG (1:150, Jackson Immunoresearch). Images for NFATc1-GFP, NFATc3-GFP and nuclear H2B-RFP were collected with a BioRad Laboratories confocal microscope (Radiance 2100 MP equipped with an argon laser for 488 nm excitation of GFP fluorescence, a Nikon 60x/1.4 Plan Apo objective, a 500 DCLPXR beamsplitter and HQ515/30 emission filter; all filters were from Chroma Technology Corp.) using Lasersharp 2000 software (BioRad). Endogenous NFATc1 was detected in transverse cryosections of rat muscles using monoclonal anti-NFATc1(7A6) antibody (1:500, Santa Cruz) and either biotinylated goat anti-mouse IgG (1:200, Vector) for DAB immunohistochemistry, or Cy3-conjugated rabbit anti-mouse IgG (1:500, Jackson Immunoresearch) for immunofluorescent detection. DAPI staining was used to identify the nuclei within muscle fibers. Images for endogenous NFATc1 were collected with a DMR epifluorescence microscope equipped with a DC100 digital charge-coupled device camera by using DC Viewer software (Leica, Milan). All images were analyzed using Image-J software (NIH image) and mean nuclear/cytoplasmic fluorescence was quantified based on the ratio of nuclear to cytoplasmic fluorescence for each muscle fiber measured (Fig. 5).

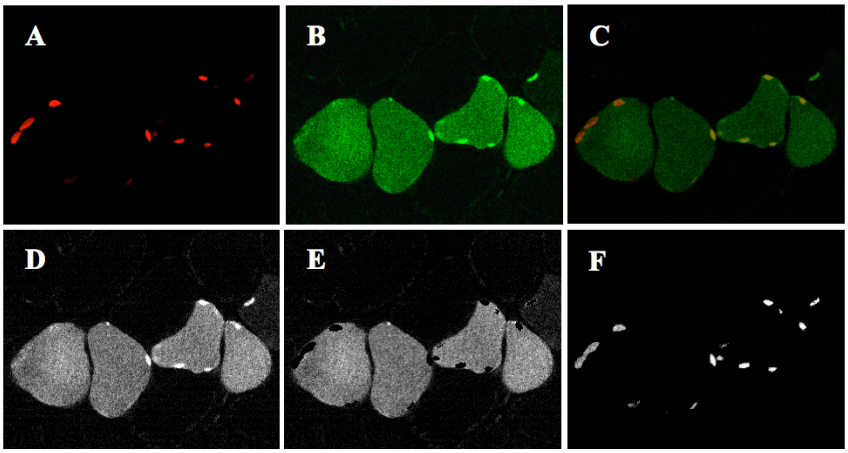


Figure 5. Quantifying cytoplasmic and nuclear fluorescence in transfected fibers using ImageJ (A) Image of transfected fibers expressing H2B-RFP are used to mark myonuclei of the transfected region. (B) Images of transfected fibers expressing NFAT-GFP fusion protein are taken at the same focal plane as nuclei. (C) Merging red and green fields allow for the identification of myonuclei within specific muscle fibers. (D) Green field images of the transfected fibers are converted to grayscale. (E) The red nuclear field is substracted from the grayscale image obtaining the cytoplasmic regions. Cytoplasmic area of each fiber is then traced with Image-J software (NIH Image). Cytoplasmic fluorescence is calculated as the mean pixel intensity of the cytoplasm for each individual fiber. Image E is then subtracted from image D, obtaining only the nuclear regions (F). Nuclear fluorescence for each fiber is then calculated by tracing all the previously identified myonuclei within the confines of each muscle fiber, giving a value of mean pixel intensity for the myonuclei of each fiber. A ratio of mean nuclear/cytoplasmic fluorescence is thus calculated for each fiber.

8. Dual Luciferase Assay

NFAT sensor transcriptional activity was determined using the Dual-Luciferase Reporter Assay Kit (Promega). Briefly, 7 days after cotransfection of NFAT sensor and reference reporter (IL-4-NFAT + RN-TK) muscles were collected at ZT 0, 4, 8, 12, 16, and 20. Frozen muscles were minced with pestle and mortar in N_2 liquid and resuspended in 2.5μL/mg passive lysis buffer. After centrifugation at 12,000g for 30 min, luciferase activity of supernatant was measured with a previously calibrated FD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Protein concentration of the supernatant was determined using the Bradford method with BSA as standard for the calibration curve. Results are expressed as the mean ratio of firefly luciferase to Renilla luciferase ± SEM.

9. Quantitative Real-Time PCR

Total RNA was prepared from tissues using the Promega SV Total RNA Isolation kit. Complimentary DNA generated with Invitrogen Superscript III reverse transcriptase was analyzed by quantitative real-time RT-PCR using the QIAGEN Quantitect SYBR Green PCR kit. All data were normalized to corresponding *β-actin* expression. Each quantitative real-time RT-PCR was performed using the ABI PRISM 7000 (Applied Biosystems) and primer sets were designed with Primer Express software (Applied Biosystems) unless otherwise specified.

Table 1. Primers for SYBR Green Real-Time RT-PCR

Gene	Primers	Source
mBmal1	5'-GCA GTG CCA CTG ACT ACC AA-3' 3'-TCC TGG ACA TTG CAT TGC AT-5'	(Yamamoto *et al.*, 2005)
mPer1	5'-GAA AGA AAC CTC TGG CTG TTC CT-3' 5'-GCT GAC GAC GGA TCT TTC TTG-3'	(Yamamoto, Nakahata et al. 2005)
mPer2	5'-GTC TTG ACC TCC ATG AAA CCT C-3' 5'-TGC TTG GGA GAT ACA ATC ACA C-3'	
mβ-Actin	5'-CAA ACA TCC CCC AAA GTT CTA C-3' 5'-TGA GGG ACT TCC TGT AAC CAC T-3'	

Specificity of gene amplification was confirmed by analyzing the dissociation curve with ABI PRISM 7000 SDS software (Applied Biosystems). For a 20-µl PCR reaction 10µl of 1:10 diluted cDNA template was mixed with the forward and reverse primers to a final concentration of 300 nM each and

9.6 µl of Quantitect SYBR Green PCR Master Mix (Qiagen). The reaction was first incubated at 50°C for 2 min, then at 95°C for 10 min, followed by 40 cycles of 94°C for 20 sec and either 58°C (*Bmal1* and *β-Actin*) or 60°C (*Per1* and *Per2*) for 1 min. Each gene-specific PCR was performed in quadruplicate.

10. Immunohistochemical detection of tyrosine hydroxylase

Sympathetic fibers were identified in cryosections of skeletal muscle by immunofluorescent labeling of tyrosine hydroxylase (TH), the first enzyme in catecholamine biosynthesis (Fig. 6). Successful chemical sympathectomy was confirmed by the absence of TH-positive sympathetic fibers in skeletal muscles of 6-OHDA treated animals. Normal TH staining of perivascular nerve tracts was detected only in vehicle-injected control animals using polyclonal rabbit anti-TH (1:400, Chemicon).

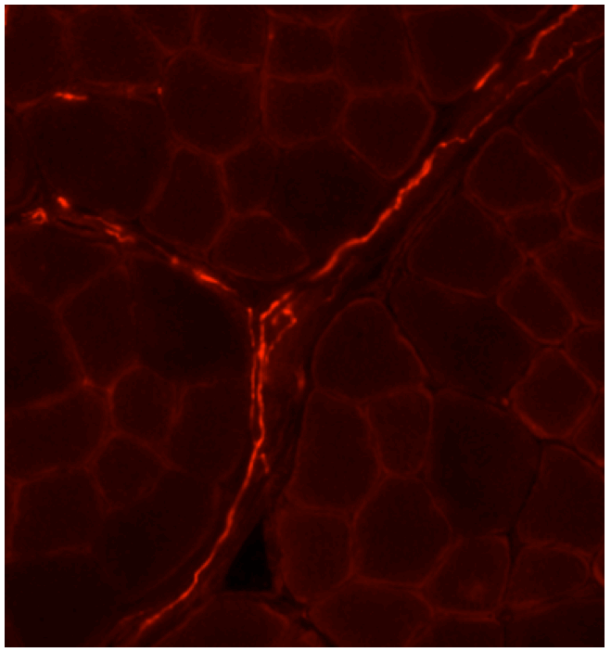


Figure 6. Representative immunofluorescent staining of sympathetic nerve fibers in transverse sections of mouse tibialis anterior using rabbit anti-TH antibody. Notice the perivascular route of the brightly labeled sympathetic fibers

RESULTS

1. Circadian rhythms of NFAT nucleocytoplasmic shuttling and transcriptional activity are nerve activity-dependent

1.1 Transfected NFATc1- and NFATc3-GFP fusion proteins exhibit circadian rhythms of nuclear localization that are more pronounced in slow muscles than in fast muscles

The calcium-dependent phosphatase calcineurin, and its downstream effectors the transcription factors of the NFAT family, are involved in the nerve-activity dependent regulation of fiber-type specific gene programs in skeletal muscle (Schiaffino & Serrano, 2002). One member of the NFAT family, NFATc1, was shown to be a nerve activity sensor *in vivo* (McCullagh *et al.*, 2004; Tothova *et al.*, 2006), making it a useful tool to monitor activity levels of individual muscle fibers *in vivo*. In the absence of nerve impulses NFATc1 resides predominantly in the cytoplasm in a phosphorylated state. A slow-type nerve stimulation pattern leads to the calcineurin-dependent dephosphorylation and rapid nuclear accumulation of NFAT in the myofibers. We wondered if this activity-dependent transcription factor also showed rhythmic nucleocytoplasmic shuttling *in vivo* in response to circadian rhythms of locomotor activity. In order to address this question we studied the circadian localization of NFAT transcription factors in both slow and fast adult mouse skeletal muscles transfected with constructs coding for NFATc1- and NFATc3-GFP fusion proteins (Fig. 7, top and bottom left). Animals were sacrificed every 4 hours for a 24-hour period, and the ratio of nuclear to cytoplasmic GFP fluorescence within individual transfected myofibers was analyzed. As shown in Figure 6, both NFATc1 and NFATc3 exhibit a significantly greater nuclear localization in slow muscle fibers at night (ZT16), corresponding with the onset of locomotor activity, than during the day (ZT4) when the animals are normally at rest. Quantification of the nuclear to cytoplasmic fluorescence (Fig. 7, top and bottom right, see Fig. 5) confirmed that there is a robust accumulation of both NFATc1 and NFATc3 isoforms in the myonuclei of the soleus starting immediately after the beginning of the dark phase (ZT12), with a clear peak at ZT16. As expected, the overall level of nuclear NFAT in the fast TA of the same animals remained relatively low, confirming that the nuclear translocation of NFAT is muscle fiber-specific and reflects different levels of activation.

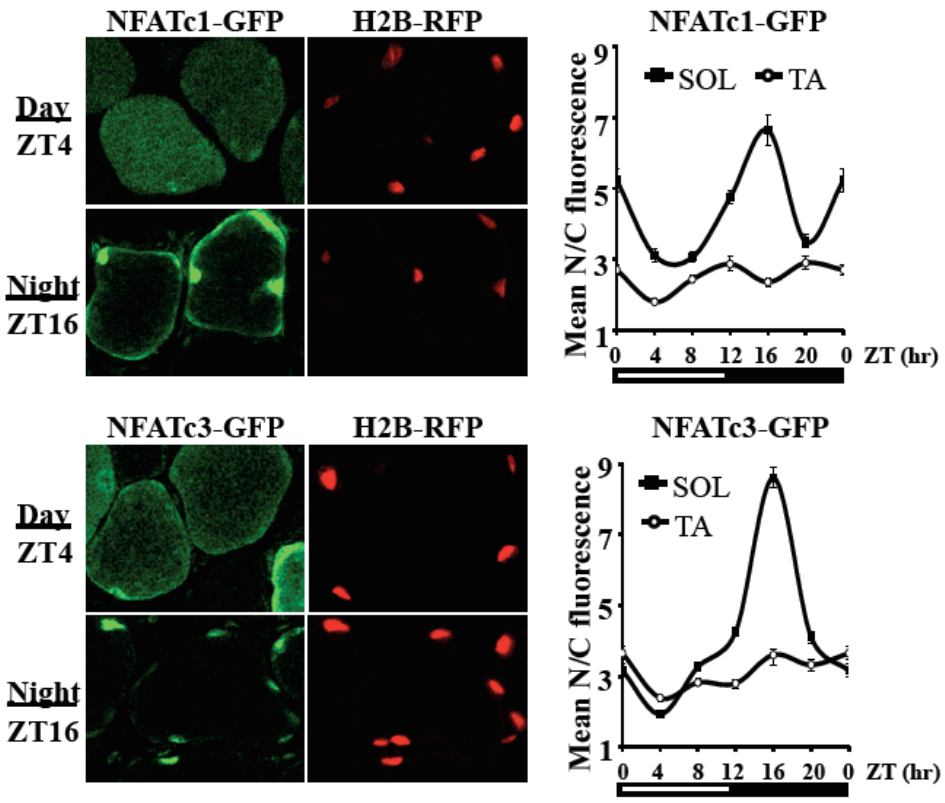


Figure 7. NFAT Circadian nucleocytoplasmic shuttling and transcriptional activity is nerve activity-dependent. Nucleocytoplasmic localization of NFATc1-GFP (top left) and NFATc3-GFP (bottom left) during the day (ZT4) and night (ZT16) revealed in transverse sections of transfected mouse soleus. There is a visibly greater nuclear localization of both NFATc1-GFP and NFATc3-GFP at night. Identification of nuclei within transfected zones is accomplished by co-transfection of a constitutively nuclear RFP construct, Histone 2B-RFP (H2B-RFP). Circadian nucleocytoplasmic shuttling of both NFATc1-GFP (top right) and NFATc3-GFP (bottom right) was quantified as the ratio of mean nuclear/cytoplasmic fluorescence (+/- SEM). Both constructs showed increased nuclear localization in transfected muscle fibers (n=6 muscles with >100 transfected fibers analyzed for each construct per time point), starting at the onset of night (lights off at ZT12) and show a peak nuclear localization 4 hours later at ZT16. The nuclear translocation pattern of NFAT-GFP corresponds with the well-documented onset of locomotor activity at the beginning of night. As expected, there is a marked difference of circadian NFAT-GFP translocation between the slow SOL and the fast TA, indicative of different circadian activity profiles. Time point ZT0 has been duplicated to better show the oscillation.

1.2 Endogenous NFATc1 shows a circadian rhythm of nuclear localization

We investigated circadian translocation of endogenous NFATc1 protein to ensure that our previous observations were not an artefact of the overexpressed NFAT-GFP fusion proteins. This time we used rat skeletal muscle due to the lack of a proper antibody for decent detection in mouse tissue. Both DAB immunohistochemistry (Fig. 8A,B) and immunofluorescence (Fig. 8C) showed a significantly greater endogenous NFATc1 nuclear signal in the slow soleus during the night (ZT16) than during the day (ZT4), confirming results observed with the transfected NFAT-GFP fusion proteins. As expected, the fast extensor digitorum longus (EDL) muscle showed a consistently weak nuclear signal at both time points (data not shown). Quantification of mean nuclear/cytoplasmic fluorescence confirmed a greater nuclear localization of endogenous NFATc1 at night (Fig. 8D).

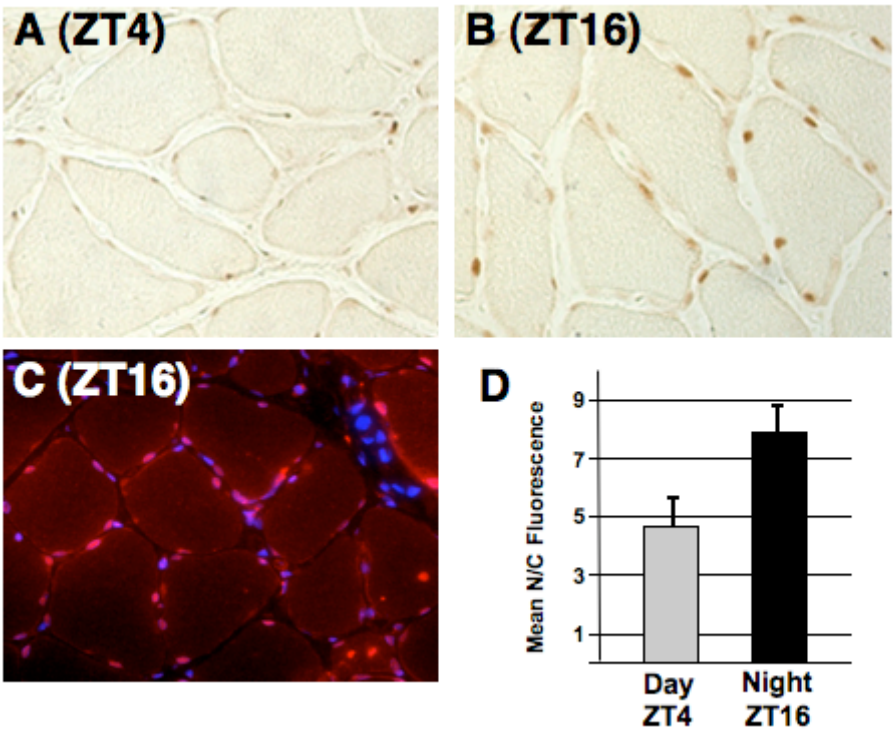


Figure 8. Endogenous NFAT also shows a circadian rhythm of nuclear localization. (A,B) Cross sections of rat soleus processed to detect NFATc1 using standard DAB immunohistochemistry. As in skeletal muscle fibers of mice transfected with NFAT-GFP fusion proteins, endogenous NFATc1 exhibits a noticeably stronger nuclear signal in tissues collected at night (B). Immunofluorescence analyses (C) and quantification of mean nuclear/cytoplasmic fluorescence (D) confirm a greater nuclear signal during the night of endogenous NFATc1. Myonuclei were identified (C) by DAPI staining.

1.3 Circadian NFAT nuclear accumulation is accompanied by transcription of NFAT targets

To assess whether the circadian nuclear accumulation of NFATc1 and NFATc3 proteins correlates with an oscillation in transcriptional activity of their targets, we transfected both slow SOL and fast TA muscles of adult mice with an NFAT reporter made by a concatamer of NFAT elements linked to a basal α-MHC promoter and luciferase (Braz *et al.*, 2003). A week after transfection, both SOL and TA muscles were collected every 4 hours for 24 hours and NFAT-dependent luciferase activity was monitored. We observed a circadian variation in luciferase activity that closely followed the cycle of nuclear NFAT accumulation with a 4-8 hour delay. In the soleus, as shown in Figure 9, the transfected NFAT reporter produced a clear peak of luciferase activity at ZT20, 8 hours after the start of increased nuclear accumulation of NFAT. This delay corresponds to the amount of time we found to be necessary for NFAT transcription factors, upon activation and nuclear translocation, to induce a measureable increase in NFAT-dependent luciferase activity (data not shown). It is also interesting to note that the fast TA also exhibited circadian variations in NFAT-dependent luciferase activity (see Fig. 16), yet 10-fold less than the slow SOL muscle. Again, it is presumed that this difference reflects the distinct activation levels of the two muscle types.

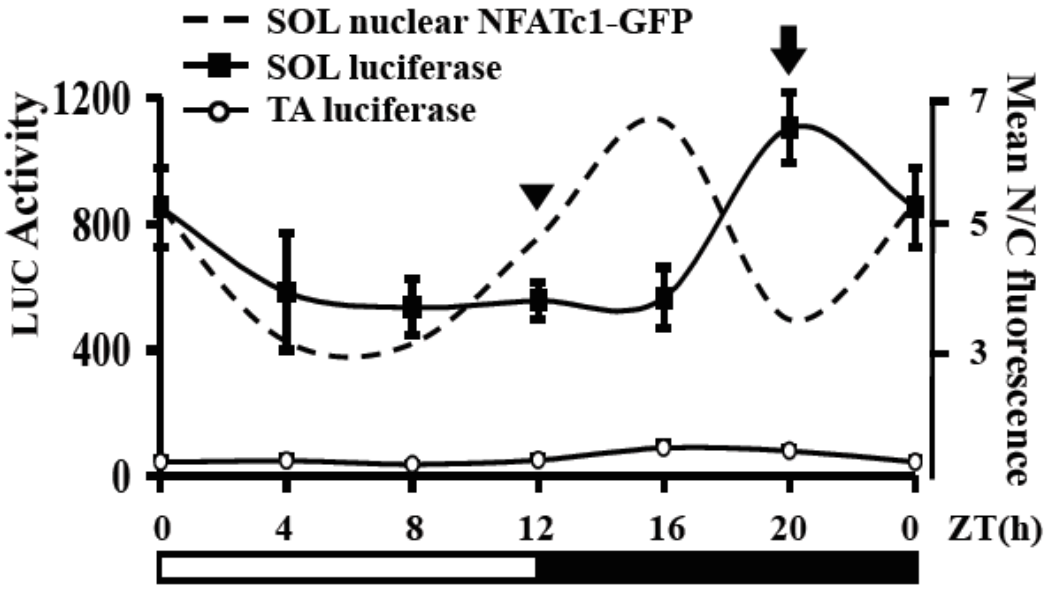


Figure 9. Circadian rhythm of NFAT transcriptional activity follows rhythmic nuclear localization. Nuclear accumulation of NFATc1-GFP (dashed line) has been superimposed to demonstrate its correlation with NFAT reporter activity. Around 8 hours after the observed start of increased nuclear accumulation of NFAT at ZT12 (arrowhead), we detected a peak (arrow) of luciferase activity from the transfected NFAT reporter (IL-4-NFAT, n=5-6 per time point) at ZT20. Similar to previous reports and the differences we observed in rhythmic nucleocytoplasmic shuttling of NFAT isoforms, TA did not show a comparable scale of luciferase production to SOL, suggesting that the rhythmic transcription of NFAT targets in skeletal muscle is muscle fiber type/motor innervation-specific. Time point ZT0 has been duplicated to better show the oscillation.

1.4 Rhythmic NFAT nuclear localization is abolished after motor denervation

To investigate whether the observed circadian rhythms of NFAT nuclear accumulation and transcriptional activity are nerve activity-dependent, we transfected mouse soleus with constructs for either NFATc1- or NFATc3-GFP fusion proteins and collected tissues at the previously established peak (ZT16) and trough (ZT4) time points for nuclear NFAT localization in the soleus. Unilateral transection of the sciatic nerve was performed 12 hours prior to sacrifice in order to eliminate motor innervation in only one hindlimb, leaving innervation of the contralateral leg intact as a control. As shown in Figure 10, the normal peak of nuclear NFATc1 and -c3 accumulation seen during the night (ZT16) in the innervated SOL (inn) is completely absent in the denervated SOL (den) of the same animals, with the peak being reduced to mere basal levels observed during the day (ZT4).

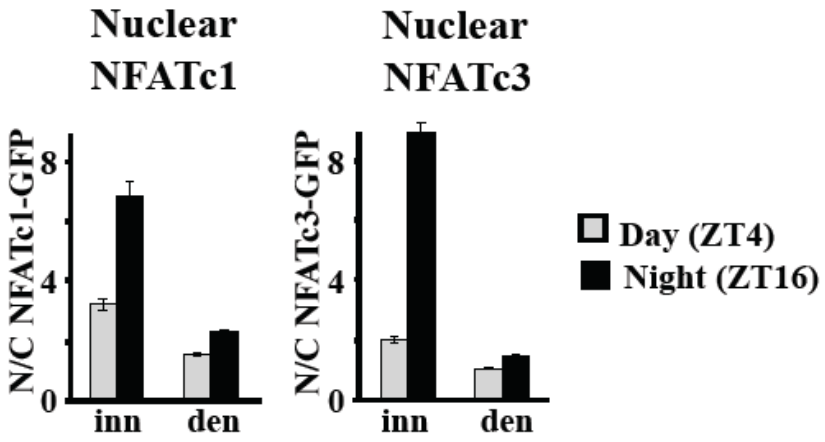


Figure 10. Circadian rhythm of NFAT nuclear translocation is nerve activity-dependent. The normal circadian rhythm of increased nuclear localization of NFAT in mouse SOL during the night (inn) is significantly reduced in the contralateral SOL following 12hours of motor denervation (den). Again, nuclear localization was quantified as the ratio of mean nuclear/cytoplasmic fluorescence (+/- SEM; n=4 muscles/group/time point, with n>100 transfected fibers analysed for each group/time point). In mouse SOL transfected with constructs for either NFATc1- or NFATc3-GFP fusion proteins, unilateral transection of the sciatic nerve 12 hours prior to tissue collection severely diminished the previously observed peak of nuclear localization at night (ZT16) to mere basal levels observed during the day (ZT4).

2. Clock gene expression in slow and fast skeletal muscle

2.1 Clock gene expression in fast and slow skeletal muscle oscillates in phase with other peripheral tissues

Previous analyses of clock gene expression in mouse skeletal muscles were performed in fast muscles (Yang *et al.*, 2006; McCarthy *et al.*, 2007). Therefore, we first asked whether the core clock genes are regulated differently in fast and slow skeletal muscle, two tissues with very different activity levels and metabolic requirements. In order to answer this question we examined the circadian expression levels of the canonical clock genes *Bmal1*, *Per1* and *Per2* in the fast TA and slow SOL muscle compared to the liver of mice kept in *ad libitum* feeding conditions. Mice were sacrificed at 4 hr intervals for 24 hours and circadian gene expression levels were determined by quantitative real-time RT-PCR normalizing for *β-actin*. In both slow and fast skeletal muscle *Bmal1* expression began to increase starting at ZT12, reaching a peak at around ZT20 where it remained relatively high in the soleus compared to TA until around ZT4 (Fig. 11, top). This was 4 hours after Per1 and Per2 expression started to increase at around ZT0, reaching peak expression levels at ZT12 and ZT16 respectively. Consistent with previous reports (Yang *et al.*, 2006), a fairly strict phase coherence was observed between expression levels in skeletal muscle and liver in mice kept in *ad libitum* feeding conditions.

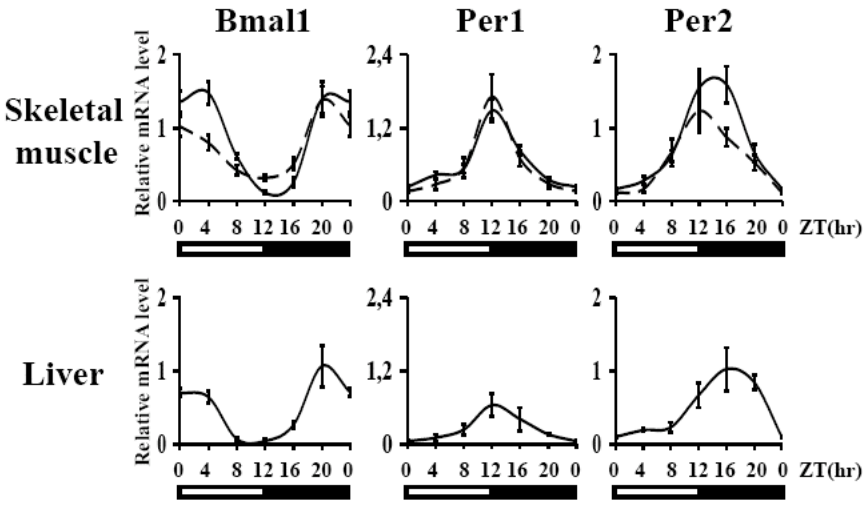


Figure 11. The skeletal muscle circadian oscillator cycles in phase with the liver in *ad libitum* feeding conditions. Mean circadian expression levels (+/-SEM) of the canonical clock genes *Bmal1*, *Per1* and *Per2* in both slow soleus (Top row, solid line) and fast tibialis anterior (Top row, dashed line) (n=5-6 per time point for each skeletal muscle) and liver (bottom row; n=3 per time point) were revealed by quantitative real-time RT-PCR. Notice each gene cycles in phase in both skeletal muscles and the liver in *ad libitum* feeding conditions. Results of each sample were normalized against corresponding *β-actin* expression levels. Time point ZT0 has been duplicated to better show the oscillation.

2.2 Rhythmic clock gene expression in skeletal muscle is not activity dependent

Is the cyclic expression of clock genes in hindlimb muscle also regulated by locomotor activity? To answer this question, we performed unilateral sciatic nerve transection on mice for either 12 hrs (short-term) or 7 days (long-term), leaving the innervation of the contralateral leg intact as a control. The expression of the clock genes in the denervated SOL muscle was investigated at the previously established time points for maximum and minimum gene expression, i.e. ZT0 and ZT12. As shown in Figure 12, all three clock genes continue to show the diurnal variations observed under normal conditions even after 12 hrs of denervation. To ensure that this lack of change was not due to the relatively short period of denervation, we also analyzed gene expression levels after 7 days of denervation, and even this long-term denervation protocol did not significantly influence the phase of clock gene expression at these time points (Fig. 12).

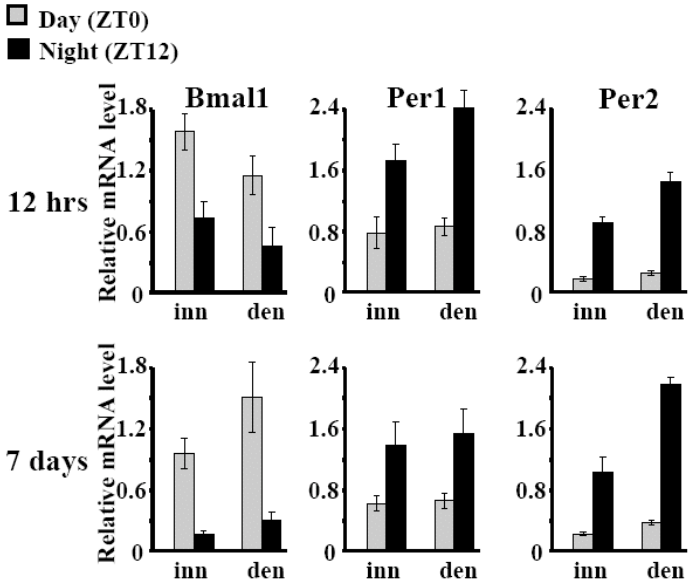


Figure 12. The skeletal muscle circadian oscillator continues to cycle in the absence of motor innervation. Mean circadian expression levels (+/-SEM) of the canonical clock genes *Bmal1*, *Per1* and *Per2* relative to *β-actin* in mouse SOL as revealed by quantitative real-time RT-PCR. For the denervated group (den), hindlimb motor denervation was performed either 12 hrs or 7 days prior to muscle collection by unilateral transection of the sciatic nerve. The innervated group (inn) was comprised of the contralateral SOL after a sham operation consisting of an identical procedure without transection of the sciatic nerve. Muscles (n=5 for each group) were collected at both ZT0 (day) and ZT12 (night), at the previously established high and low relative expression points for the selected genes (see Fig. 11).

2.3 Clock gene expression in skeletal muscle is unaltered after chemical sympathectomy

The hypothalamic-sympathetic nervous system axis has been implicated in communicating timing information from the SCN to peripheral tissues for circadian gene regulation (Terazono *et al.*, 2003). Skeletal muscles also receive inputs from the sympathetic nervous system (Barker & Saito, 1981; Rotto-Percelay *et al.*, 1992), and hindlimb muscle sympathetic innervation is not completely abolished by sciatic transection (Minokoshi *et al.*, 2002). To completely abolish sympathetic nerve activity in skeletal muscle while leaving motor innervation intact, adult mice were treated for two weeks with 6-OHDA. This particular treatment protocol (2 weeks) was selected to avoid noted side-effects reported in the literature which could potentially complicate the interpretation of our results, including significantly reduced food intake and alterations in locomotor activity (Terazono *et al.*, 2003). Sympathetic fibers were identified in skeletal muscle cryosections by the presence of tyrosine hydroxylase (TH), the first enzyme in catecholamine biosynthesis. Successful chemical sympathectomy was confirmed by the absence of TH-positive sympathetic fibers in skeletal muscles of 6-OHDA treated animals (Fig. 13). Similar to our results after motor denervation, the chemical sympathectomy had no major effect on the phase of clock gene oscillation in skeletal muscle, even though *Bmal1* expression was slightly higher, and *Per1* and *Per2* peak amplitude slightly lower (Fig. 14). A recent report investigating phase entrainment in the submaxillary gland found similar results (Vujovic *et al.*, 2008), with sympathetic denervation affecting predominantly amplitude, but not phase of clock gene expression. It was presumed that sympathetic innervation directly relays light cycle cues to peripheral tissues, and that feeding cues compete either strongly or weakly with sympathetic cues, depending on the tissue. Our results suggest that sympathetic innervation in skeletal muscle provides relatively weak entrainment signals.

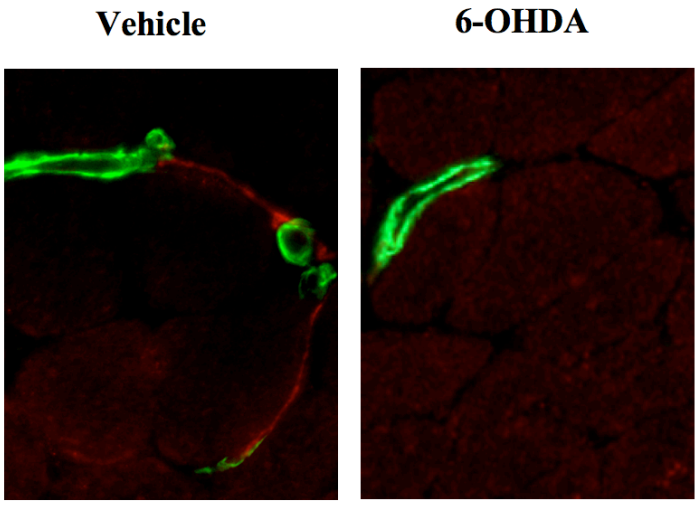
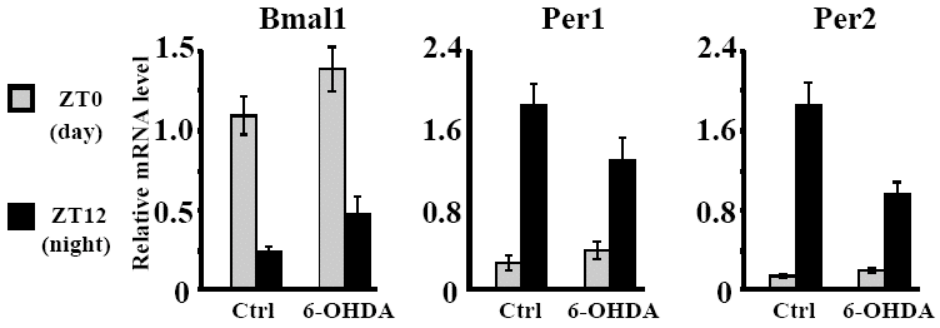


Figure 13. Peripheral sympathetic innervation of hindlimb muscles was visualized by tyrosine hydroxylase (red) immunofluorescence near blood vessels (green) in animals treated with vehicle (left), but was absent in those treated with 6-OHDA (right). Blood vessels were identified using antibodies against α-smooth muscle actin (Sigma).

Figure 14. The skeletal muscle circadian oscillator continues to cycle in the absence of sympathetic innervation. (A) Mean circadian expression levels (+/- SEM) of the canonical clock genes *Bmal1*, *Per1* and *Per2* relative to *β-actin* in mouse SOL as revealed by quantitative real-time RT-PCR. For the chemical sympathectomy group (6-OHDA), mice were treated with 6-OHDA. Muscles (n=5 for each group) were collected at both ZT0 (day) and ZT12 (night), the previously established high and low relative expression points for the selected genes (see Fig. 11).



2.4 Restricted feeding shifts the phase of rhythmic *Bmal1* expression in skeletal muscle, while *Per1* and *Per2* gene oscillation is blunted

It has been shown that feeding entrains circadian oscillators in most peripheral tissues studied to date, uncoupling them from the SCN and altering the tissue's circadian gene expression profile to coincide with food availability. To assess whether the skeletal muscle core oscillator is regulated by metabolic cues, we restricted food access to the light phase (ZT0-ZT12), while depriving the animals of food during the dark phase (ZT12-ZT0), when the animals normally eat in *ad libitum* feeding conditions. After 14 days of this restricted feeding schedule the animals were sacrificed and the circadian expression profiles of the clock genes was determined. As expected, the phases of *Per1*, *Per2* and *Bmal1* expression were shifted by about 12 hours in the liver, in agreement with previous reports (Damiola *et al.*, 2000; Stokkan *et al.*, 2001), with peak expression of *Per1* and *Per2* occurring at ZT0-4 as opposed to ZT12-16, and the start of *Bmal1* accumulation beginning at ZT4 rather than ZT16 (Fig. 15). In both the slow SOL and the fast TA, *Bmal1* showed the same phase shift as that seen in liver. *Per1* and *Per2* oscillation also showed a similar phase shift trend as that seen in the liver, but the oscillation was blunted. *Per1* in particular seemed to be at a relatively constant state of intermediate expression, even though the point of lowest expression was shifted by about 12 hours to ZT12. A similar result was recently reported to occur in brown adipose tissue after restricted feeding (Reilly *et al.*, 2008), in which *Per1* and *Per2* did not re-entrain as well as in heart and liver after restricted feeding, yet *Bmal1* phase was altered similarly in all the tissues investigated.

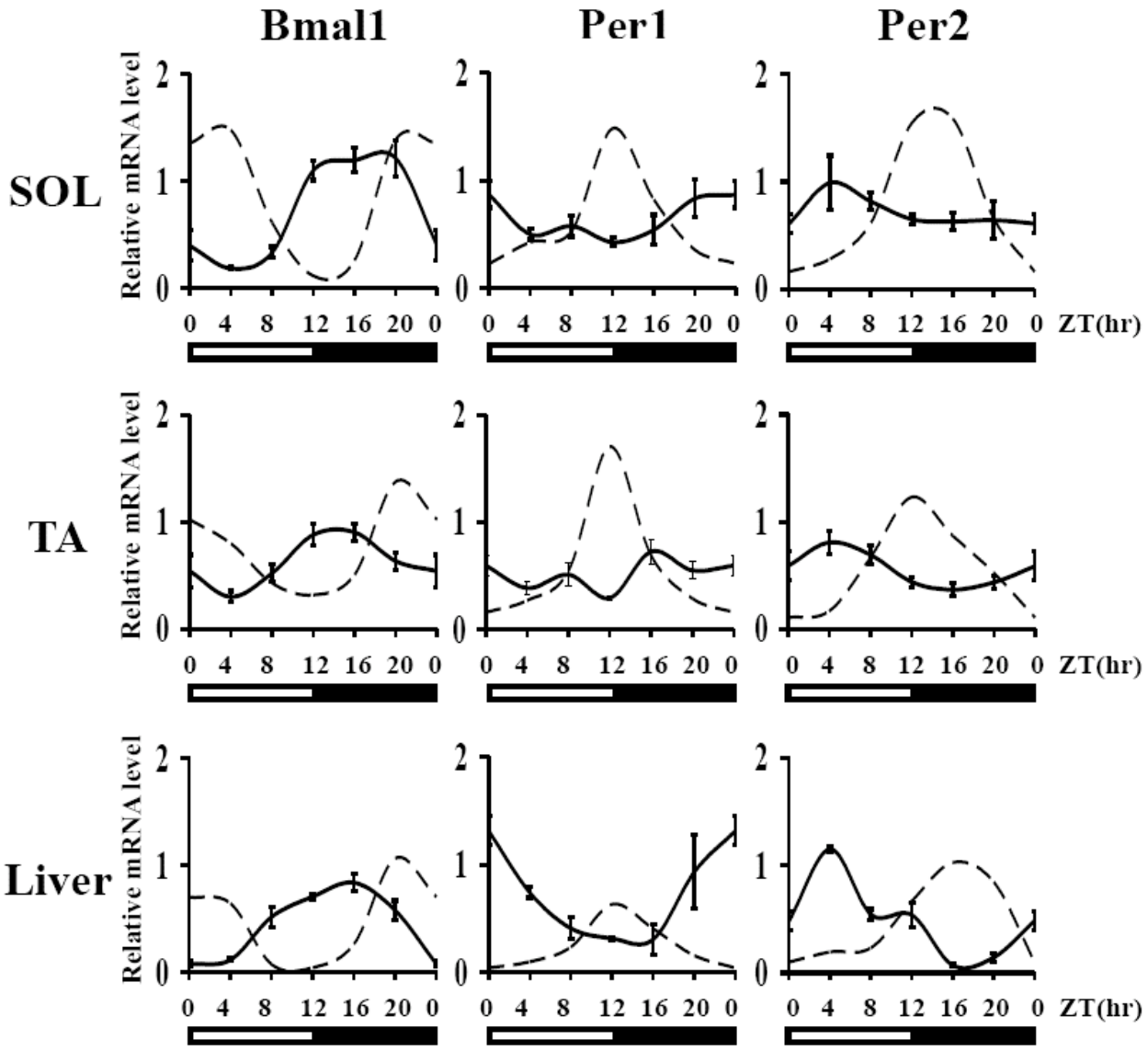


Figure 15. Restricted feeding shifts phase of rhythmic Bmal1 expression in skeletal muscle, while Per1 and Per2 gene oscillation is blunted. Circadian expression profiles of core oscillator genes in skeletal muscle are altered in response to 14 days of restricted day feeding. Notice that *Bmal1* oscillation was successfully entrained to day feeding in both slow and fast skeletal muscle as in liver compared to *ad libitum* feeding (dashed line), whereas both *Per1* and *Per2* oscillation were somewhat blunted in skeletal muscle. This suggests that signals resulting from food availability are a driving force behind *Bmal1* oscillation, whereas *Period* gene oscillation in skeletal muscle may depend on contributions from additional signals. Mean circadian expression levels (+/-SEM) were revealed by quantitative RT-PCR in both slow (SOL) and fast (TA) skeletal muscle (n=5-6 per time point) and in liver (n=3 per time point). Results of each sample were normalized against corresponding *β-actin* expression levels. Time point ZT0 has been duplicated to better show the oscillation.

DISCUSSION

Despite being one of the most abundant tissues in the body, the mechanisms regulating the circadian clock in skeletal muscle are not well established. Playing an essential role in both whole body metabolism as well as locomotor activity, it is of pivotal importance (McDearmon *et al.*, 2006) to better understand the factors regulating the skeletal muscle oscillator. Activity is thought to be important in the regulation of both the amplitude as well as the phase of the circadian clock in skeletal muscle (Zambon *et al.*, 2003; McCarthy *et al.*, 2007; Miller *et al.*, 2007), yet numerous groups have shown that the predominant entrainment signals in peripheral tissues are metabolic factors due to food availability (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Brown *et al.*, 2002). Indeed, recent studies have pointed out that skeletal muscle circadian oscillators and energy metabolism are intimately linked. Specifically, AMPK, a critical regulator of energy homeostasis, is important in the regulation of *Per1* (Vieira *et al.*, 2008), and PGC-1α, a transcriptional coactivator that regulates energy metabolism, stimulates the expression of *Bmal1* and *Rev-erbα* through coactivation of the ROR family of orphan nuclear receptors (Liu *et al.*, 2007). On the other hand, more than a third of 215 circadian genes identified in skeletal muscle exhibit peak expression at ZT18, a time when mice are most active and feeding, suggesting a possible role for activity in the regulation of clock components (McCarthy *et al.*, 2007; Miller *et al.*, 2007). Here we show that while locomotor activity does not entrain the core oscillator in skeletal muscle, it is necessary for the circadian nucleocytoplasmic shuttling of activity-dependent transcription factors such as NFAT, explaining the highest level of circadian gene expression during the active phase (McCarthy *et al.*, 2007; Miller *et al.*, 2007). In contrast, a restricted feeding schedule phase-shifted rhythmic clock gene expression in skeletal muscle suggesting that metabolic factors, not activity, entrain rhythmic clock gene expression in skeletal muscle.

NFAT nucleocytoplasmatic shuttling and transcriptional activity shows circadian rhythms that are nerve activity-dependent

We report here that nucleocytoplasmic shuttling of NFAT transcription factors shows nerve activity-dependent daily variations. The family of NFAT transcription factors consists of four different isoforms activated by the calcium-dependent phosphatase calcineurin. NFATc1 translocates rapidly from the cytoplasm to the nucleus when muscles are stimulated with a pattern simulating slow motor

neuron firing and plays an important role in the maintenance of the slow fiber (McCullagh *et al.*, 2004; Tothova *et al.*, 2006). When examining nuclear accumulation of both NFATc1 and NFATc3 using NFATc1- and NFATc3-GFP fusion proteins in the slow soleus muscle we detected a clear circadian rhythm that closely followed previously reported activation via circadian motor nerve firing rhythms (Elder & Toner, 1998). Nuclear accumulation begins around ZT12, coinciding with the onset of locomotor activity, with a clear peak at ZT16. Indeed, it was previously shown that low frequency electrical stimulation for 30 minutes is sufficient to induce a significant nuclear accumulation of NFAT (Tothova *et al.*, 2006). NFAT-dependent transcription closely follows nuclear accumulation of NFAT with a delay of 4-8 hours, corresponding to the time required for luciferase production. Even the fast TA muscle showed a small circadian increase in NFAT-dependent luciferase activity (Fig. 16), albeit on a scale about ten-fold lower than that observed in the SOL. This is consistent with the much lower activation level of motor units in fast muscles (Hennig & Lomo, 1985) and underscores the sensitivity of NFAT to activity.

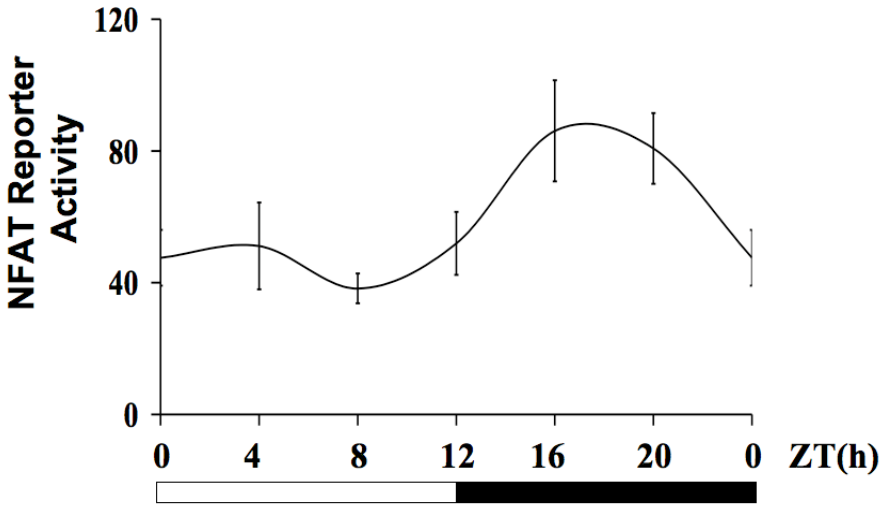


Figure 16. (compare with Fig. 9) Closer view of circadian NFAT-dependent luciferase activity in tibialis anterior (TA) transfected with an NFAT reporter (IL-4-NFAT, n=5-6 per time point) shows that fast TA also exhibits circadian variations in NFAT-dependent luciferase activity, yet on a scale 10-fold less than that detected in slow soleus.

Entrainment of the skeletal muscle core oscillator is not nerve activity-dependent

Studies have suggested that as much as 20% of the mammalian transcriptome may be under circadian regulation, yet very few of circadian genes are common among different tissues. This is thought to reflect the diversity of tissue-specific physiological processes in different tissues (Panda *et al.*, 2002; Storch *et al.*, 2002). We examined the expression of three core clock genes *Bmal1*, *Per1* and *Per2* both in slow and fast muscles since these tissues differ strongly both in metabolic profile, with fast muscles being predominately glycolytic and slow muscles oxidative, as well as activity pattern, with fast muscles used infrequently, whereas slow muscles show more tonic levels of activation (Schiaffino & Reggiani, 1996). In agreement with previous studies of fast skeletal muscle, we show here that in *ad libitum* feeding conditions the expression of the core oscillator genes *Bmal1*, *Per1* and *Per2* in liver is synchronous with their expression in both fast and slow skeletal muscles.

Since skeletal muscle is critically important for locomotor activity it has been suggested that circadian activity rhythms may be responsible for the phase-coordinated expression of rhythmic genes (Zambon *et al.*, 2003; Miller *et al.*, 2007). It was previously noted that there is a clear peak in the number of genes expressed in skeletal muscle at ZT18, 6 hours after the beginning of the activity phase (McCarthy *et al.*, 2007) and resistance exercise was shown to induce the expression of components of the core oscillator (Zambon *et al.*, 2003). Here we show that under normal conditions, activity-dependent transcription factors such as NFAT indeed show circadian rhythms of translocation (Fig. 7) leading to rhythmic increases in transcriptional activity in skeletal muscle at specific time points (Fig. 9) which coincide with activity rhythms observed by others (McCarthy *et al.*, 2007; Miller *et al.*, 2007; Almon *et al.*, 2008), yet these rhythms are completely abolished by motor denervation (Fig. 10). Conversely, motor innervation is not necessary for rhythmic expression of the core oscillator genes *Bmal1*, *Per1* and *Per2*, as both short-term (12 hrs) and long-term (7 days) denervation failed to significantly influence the phase of these genes (Fig. 12), clearly showing that motor neuron activity and core oscillator gene expression are not linked in skeletal muscle.

Recently, the sympathetic nervous system was implied to be important for the regulation of the expression of clock genes in peripheral tissues (Terazono *et al.*, 2003; Vujovic *et al.*, 2008). Sympathetic innervation is known to be of primary importance in regulating bloodflow in skeletal muscle (Marshall, 1982; Fleming *et al.*, 1989) and increased sympathetic tone is induced in response to exercise. Adrenergic signaling is also known to regulate genes important for oxidative metabolism, such as *NOR-1* (Pearen *et al.*, 2008) and *PGC-1α*, a gene which mediates expression of *Bmal1*. In

addition to motor denervation, sciatic nerve transection partially eliminates sympathetic innervation of the hindlimb. In a previous study, unilateral sciatic denervation was observed to reduce total catecholamine content of a denervated soleus to 62% of the intact, contralateral soleus, whereas combined denervation of the sciatic, femoral and obturator nerves, which together block both sympathetic and motor denervation, decreased catecholamine content to 6% of control levels (Minokoshi *et al.*, 2002). In order to completely eliminate sympathetic innervation to the hindlimb while leaving motor innervation intact, we performed a chemical sympathectomy with 6-OHDA. This treatment is drastic in that it transiently eliminates all peripheral sympathetic innervation in adults, and animals undergoing this treatment have been known to alter activity and feeding behavior soon after the treatment begins, leading to significant decreases in feeding (Terazono *et al.*, 2003) and body weight which could potentially complicate the interpretation of results. To minimize these variables we prolonged the treatment protocol to two weeks, which was sufficient for body weight of the treated animals to return to control levels (Fig. 17). We saw no significant changes in the phase of the core oscillator gene expression, even though peak expression of Per1 and Per2 was somewhat reduced. This reduction was also found in the liver after performing a chemical sympathectomy with 6-OHDA (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Terazono *et al.*, 2003).

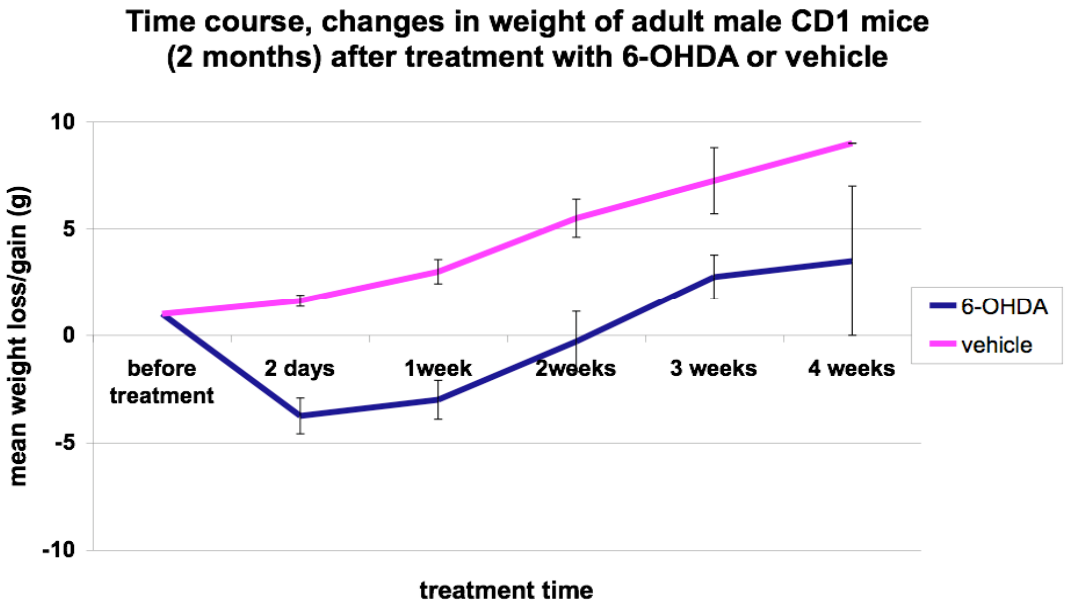


Figure 17. Timecourse of mean weight loss/gain of 2-month old male CD1 mice during long-term weekly 6-OHDA treatments. Notice that 2 weeks is sufficient for recovery from the initial weight loss due to 6-OHDA treatment.

Feeding schedule entrains the skeletal muscle core oscillator

It has been shown in some peripheral tissues, such as liver, kidney, heart, pancreas and some brain regions, that forced changes in feeding patterns can uncouple peripheral oscillators from the SCN, shifting the phase of peripheral oscillator gene expression (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). When food is made available only during the light phase, nocturnal rodents are forced to eat when they would normally be asleep. This was observed to shift the phase of core oscillator gene expression in various peripheral tissues without affecting the SCN. The fact that the oscillator in skeletal muscle is also strongly influenced by feeding time is not surprising considering the important role of skeletal muscle in whole-body metabolism.

Plasma glucose concentrations and glucose uptake are known to follow circadian changes, even in fasting conditions, starting to rise just before the onset of activity (Pauly & Scheving, 1967; Carroll & Nestel, 1973) and increasing during the active period. Accounting for 40% of the body weight, skeletal muscle is the major site of post-prandial glucose clearance and is also involved in fatty acid uptake. The transcriptional co-activator PGC-1α is known to modulate mitochondrial biogenesis and oxidative metabolism (Wu *et al.*, 1999; Lin *et al.*, 2005), and shows diurnal variations in expression levels that are antiphase to plasma glucose concentrations (Liu *et al.*, 2007). This is possibly due to the fact that in periods of low energy sources, AMPK is activated leading to coactivation of *SIRT1* and *PGC-1α* (Fulco *et al.*, 2008). Injection of the AMPK-activating drug metformin leads to a similar phase shift of *Bmal1* expression to that seen after restricted feeding (Um *et al.*, 2007). Furthermore, PGC-1α leads to increased Bmal1 production by co-activation of transcription factors RORα and RORγ (Liu *et al.*, 2007), while SIRT1-activation, via deacetylation of Per2, leads to transcription of several core clock genes (Asher *et al.*, 2008; Nakahata *et al.*, 2008). In addition to post-translational events, the timing of these transcriptional events are also important factors determining the phase of the cycle, although *SIRT1* has been mainly linked to the amplitude of the cycle.

Restricted feeding phase-shifted *Bmal1* expression in skeletal muscle (Fig. 14), corresponding to the shift seen in liver, whereas the oscillation of the *Period* genes was blunted. This might be because the timeframe we used (14 days) was not long enough to establish *Per* gene oscillation, as it is known that entrainment proceeds with different kinetics in different tissues (Damiola *et al.*, 2000; Guo *et al.*, 2005). It could also be that additional factors are involved in *Period* gene regulation in skeletal muscle. In the liver, *Per2* expression is regulated independently from *Bmal1* expression both by intrinsic as well as systemic cues (Kornmann *et al.*, 2007). Glucose in particular is known to

downregulate expression of *Per1* and *Per2* (Hirota *et al.*, 2002). A similar result of blunted *Per1* and *Per2* expression after restricted feeding was recently reported to occur in brown adipose tissue (Reilly, Curtis et al. 2008) making it tempting to speculate that attenuated *Per1* and *Per2* oscillation in these tissues after restricted feeding is a result of common regulatory elements. Both brown adipose tissue and skeletal muscle are effectors for non-shivering thermogenesis activated by the sympathetic nervous system. Further exploration of this relationship is warranted, and will no doubt offer useful insights into the underpinnings of skeletal muscle physiology and whole-body fuel economy, especially in the midst of a worldwide obesity epidemic.

REFERENCES

Albrecht U, Bordon A, Schmutz I & Ripperger J. (2007). The multiple facets of Per2. *Cold Spring Harb Symp Quant Biol* **72,** 95-104.

Almon RR, Yang E, Lai W, Androulakis IP, Ghimbovschi S, Hoffman EP, Jusko WJ & Dubois DC. (2008). Relationships between Circadian Rhythms and Modulation of Gene Expression by Glucocorticoids in Skeletal Muscle. *American journal of physiology*.

Amasaki Y, Adachi S, Ishida Y, Iwata M, Arai N, Arai K & Miyatake S. (2002). A constitutively nuclear form of NFATx shows efficient transactivation activity and induces differentiation of CD4(+)CD8(+) T cells. *The Journal of biological chemistry* **277,** 25640-25648.

Aschoff J. (1991). Activity in anticipation and in succession of a daily meal. *Bollettino della Societa italiana di biologia sperimentale* **67,** 213-228.

Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, Kreppel F, Mostoslavsky R, Alt FW & Schibler U. (2008). SIRT1 regulates circadian clock gene expression through PER2 deacetylation. *Cell* **134,** 317-328.

Bae K, Lee K, Seo Y, Lee H, Kim D & Choi I. (2006). Differential effects of two period genes on the physiology and proteomic profiles of mouse anterior tibialis muscles. *Mol Cells* **22,** 275-284.

Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schutz G & Schibler U. (2000). Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science (New York, NY* **289,** 2344-2347.

Balsalobre A, Damiola F & Schibler U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93,** 929-937.

Balsalobre A, Marcacci L & Schibler U. (2000). Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Curr Biol* **10,** 1291-1294.

Barker D & Saito M. (1981). Autonomic innervation of receptors and muscle fibres in cat skeletal muscle. *Proc R Soc Lond B Biol Sci* **212,** 317-332.

Baron R, Janig W & Kollmann W. (1988). Sympathetic and afferent somata projecting in hindlimb nerves and the anatomical organization of the lumbar sympathetic nervous system of the rat. *The Journal of comparative neurology* **275,** 460-468.

Bartness TJ, Song CK & Demas GE. (2001). SCN efferents to peripheral tissues: implications for biological rhythms. *Journal of biological rhythms* **16,** 196-204.

Beaver LM, Gvakharia BO, Vollintine TS, Hege DM, Stanewsky R & Giebultowicz JM. (2002). Loss of circadian clock function decreases reproductive fitness in males of Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 2134-2139.

Bombardi C, Grandis A, Chiocchetti R, Bortolami R, Johansson H & Lucchi ML. (2006). Immunohistochemical localization of alpha(1a)-adrenoreceptors in muscle spindles of rabbit masseter muscle. *Tissue & cell* **38,** 121-125.

Bray MS, Shaw CA, Moore MW, Garcia RA, Zanquetta MM, Durgan DJ, Jeong WJ, Tsai JY, Bugger H, Zhang D, Rohrwasser A, Rennison JH, Dyck JR, Litwin SE, Hardin PE, Chow CW, Chandler MP, Abel ED & Young ME. (2008). Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression. *Am J Physiol Heart Circ Physiol* **294,** H1036-1047.

Braz JC, Bueno OF, Liang Q, Wilkins BJ, Dai YS, Parsons S, Braunwart J, Glascock BJ, Klevitsky R, Kimball TF, Hewett TE & Molkentin JD. (2003). Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling. *J Clin Invest* **111,** 1475-1486.

Brown SA, Zumbrunn G, Fleury-Olela F, Preitner N & Schibler U. (2002). Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Curr Biol* **12,** 1574-1583.

Buijs RM, Wortel J, Van Heerikhuize JJ, Feenstra MG, Ter Horst GJ, Romijn HJ & Kalsbeek A. (1999). Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. *Eur J Neurosci* **11,** 1535-1544.

Carroll KF & Nestel PJ. (1973). Diurnal variation in glucose tolerance and in insulin secretion in man. *Diabetes* **22,** 333-348.

Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Zhu W, Bassel-Duby R & Williams RS. (1998). A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev* **12,** 2499-2509.

Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F & Schibler U. (Balsalobre #64). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* **14,** 2950-2961.

Davidson AJ. (2006). Search for the feeding-entrainable circadian oscillator: a complex proposition. *American journal of physiology* **290,** R1524-1526.

Davidson AJ, Yamazaki S, Arble DM, Menaker M & Block GD. (2008). Resetting of central and peripheral circadian oscillators in aged rats. *Neurobiol Aging* **29,** 471-477.

Dodd AN, Salathia N, Hall A, Kevei E, Toth R, Nagy F, Hibberd JM, Millar AJ & Webb AA. (2005). Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science (New York, NY* **309,** 630-633.

Downes M, Carozzi AJ & Muscat GE. (1995). Constitutive expression of the orphan receptor, Rev-erbA alpha, inhibits muscle differentiation and abrogates the expression of the myoD gene family. *Mol Endocrinol* **9,** 1666-1678.

Elder GC & Toner LV. (1998). Muscle shortening induced by tenotomy does not reduce activity levels in rat soleus. *The Journal of physiology* **512 (Pt 1),** 251-265.

Elenkov IJ, Wilder RL, Chrousos GP & Vizi ES. (2000). The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. *Pharmacological reviews* **52,** 595-638.

Ellingsen T, Bener A & Gehani AA. (2007). Study of shift work and risk of coronary events. *J R Soc Health* **127,** 265-267.

English AW & Schwartz G. (2002). Development of sex differences in the rabbit masseter muscle is not restricted to a critical period. *J Appl Physiol* **92,** 1214-1222.

Fleming BP, Gibbins IL, Morris JL & Gannon BJ. (1989). Noradrenergic and peptidergic innervation of the extrinsic vessels and microcirculation of the rat cremaster muscle. *Microvasc Res* **38,** 255-268.

Freyssenet D. (2007). Energy sensing and regulation of gene expression in skeletal muscle. *J Appl Physiol* **102,** 529-540.

Fulco M, Cen Y, Zhao P, Hoffman EP, McBurney MW, Sauve AA & Sartorelli V. (2008). Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. *Dev Cell* **14,** 661-673.

Grassi G & Esler M. (1999). How to assess sympathetic activity in humans. *Journal of hypertension* **17,** 719-734.

Green CB, Takahashi JS & Bass J. (2008). The meter of metabolism. *Cell* **134,** 728-742.

Guo H, Brewer JM, Champhekar A, Harris RB & Bittman EL. (2005). Differential control of peripheral circadian rhythms by suprachiasmatic-dependent neural signals. *Proceedings of the National Academy of Sciences of the United States of America* **102,** 3111-3116.

Guo H, Brewer JM, Lehman MN & Bittman EL. (2006). Suprachiasmatic regulation of circadian rhythms of gene expression in hamster peripheral organs: effects of transplanting the pacemaker. *J Neurosci* **26,** 6406-6412.

Hastings MH, Reddy AB & Maywood ES. (2003). A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat Rev Neurosci* **4,** 649-661.

Hein L. (2006). Adrenoceptors and signal transduction in neurons. *Cell and tissue research* **326,** 541-551.

Hennig R & Lomo T. (1985). Firing patterns of motor units in normal rats. *Nature* **314,** 164-166.

Henriksson J, Svedenhag J, Richter EA, Christensen NJ & Galbo H. (1985). Skeletal muscle and hormonal adaptation to physical training in the rat: role of the sympatho-adrenal system. *Acta physiologica Scandinavica* **123,** 127-138.

Hirota T, Okano T, Kokame K, Shirotani-Ikejima H, Miyata T & Fukada Y. (2002). Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. *The Journal of biological chemistry* **277,** 44244-44251.

Hodgson JA, Wichayanuparp S, Recktenwald MR, Roy RR, McCall G, Day MK, Washburn D, Fanton JW, Kozlovskaya I & Edgerton VR. (2001). Circadian force and EMG activity in hindlimb muscles of rhesus monkeys. *J Neurophysiol* **86,** 1430-1444.

Ishida N, Kaneko M & Allada R. (1999). Biological clocks. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 8819-8820.

Kalsbeek A, Kreier F, Fliers E, Sauerwein HP, Romijn JA & Buijs RM. (2007). Minireview: Circadian control of metabolism by the suprachiasmatic nuclei. *Endocrinology* **148,** 5635-5639.

Kardos A, Taylor DJ, Thompson C, Styles P, Hands L, Collin J & Casadei B. (2000). Sympathetic denervation of the upper limb improves forearm exercise performance and skeletal muscle bioenergetics. *Circulation* **101,** 2716-2720.

Karlsson B, Knutsson A & Lindahl B. (2001). Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27,485 people. *Occup Environ Med* **58,** 747-752.

Karlsson J & Smith HJ. (1983). The effect of lumbar sympathectomy on fiber composition, contractility of skeletal muscle and regulation of central circulation in dogs. *Acta physiologica Scandinavica* **119,** 1-6.

Kirschbaum BJ, Simoneau JA, Bar A, Barton PJ, Buckingham ME & Pette D. (1989). Chronic stimulation-induced changes of myosin light chains at the mRNA and protein levels in rat fast-twitch muscle. *European journal of biochemistry / FEBS* **179,** 23-29.

Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV & Antoch MP. (2006). Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes Dev* **20,** 1868-1873.

Kornmann B, Schaad O, Bujard H, Takahashi JS & Schibler U. (2007). System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. *PLoS Biol* **5,** e34.

Kornmann B, Schaad O, Reinke H, Saini C & Schibler U. (2007). Regulation of circadian gene expression in liver by systemic signals and hepatocyte oscillators. *Cold Spring Harb Symp Quant Biol* **72,** 319-330.

Krieger DT, Hauser H & Krey LC. (1977). Suprachiasmatic nuclear lesions do not abolish food-shifted circadian adrenal and temperature rhythmicity. *Science (New York, NY* **197,** 398-399.

Kubis HP, Scheibe RJ, Meissner JD, Hornung G & Gros G. (2002). Fast-to-slow transformation and nuclear import/export kinetics of the transcription factor NFATc1 during electrostimulation of rabbit muscle cells in culture. *The Journal of physiology* **541,** 835-847.

Lin J, Handschin C & Spiegelman BM. (2005). Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metab* **1,** 361-370.

Liu C, Li S, Liu T, Borjigin J & Lin JD. (2007). Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism. *Nature* **447,** 477-481.

Liu Y & Schneider MF. (1998). Fibre type-specific gene expression activated by chronic electrical stimulation of adult mouse skeletal muscle fibres in culture. *The Journal of physiology* **512 (Pt 2),** 337-344.

Lynch GS & Ryall JG. (2008). Role of beta-adrenoceptor signaling in skeletal muscle: implications for muscle wasting and disease. *Physiological reviews* **88,** 729-767.

Marshall JM. (1982). The influence of the sympathetic nervous system on individual vessels of the microcirculation of skeletal muscle of the rat. *The Journal of physiology* **332,** 169-186.

Maspers M, Ekelund U, Bjornberg J & Mellander S. (1991). Protective role of sympathetic nerve activity to exercising skeletal muscle in the regulation of capillary pressure and fluid filtration. *Acta physiologica Scandinavica* **141,** 351-361.

McCarthy JJ, Andrews JL, McDearmon EL, Campbell KS, Barber BK, Miller BH, Walker JR, Hogenesch JB, Takahashi JS & Esser KA. (2007). Identification of the circadian transcriptome in adult mouse skeletal muscle. *Physiol Genomics* **31,** 86-95.

McCullagh KJ, Calabria E, Pallafacchina G, Ciciliot S, Serrano AL, Argentini C, Kalhovde JM, Lomo T & Schiaffino S. (2004). NFAT is a nerve activity sensor in skeletal muscle and controls activity-dependent myosin switching. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 10590-10595.

McDearmon EL, Patel KN, Ko CH, Walisser JA, Schook AC, Chong JL, Wilsbacher LD, Song EJ, Hong HK, Bradfield CA & Takahashi JS. (2006). Dissecting the functions of the mammalian clock protein BMAL1 by tissue-specific rescue in mice. *Science (New York, NY* **314,** 1304-1308.

McNamara P, Seo SB, Rudic RD, Sehgal A, Chakravarti D & FitzGerald GA. (2001). Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. *Cell* **105,** 877-889.

Meissner JD, Gros G, Scheibe RJ, Scholz M & Kubis HP. (2001). Calcineurin regulates slow myosin, but not fast myosin or metabolic enzymes, during fast-to-slow transformation in rabbit skeletal muscle cell culture. *The Journal of physiology* **533,** 215-226.

Miller BH, McDearmon EL, Panda S, Hayes KR, Zhang J, Andrews JL, Antoch MP, Walker JR, Esser KA, Hogenesch JB & Takahashi JS. (2007). Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 3342-3347.

Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D & Kahn BB. (2002). Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* **415,** 339-343.

Moser M, Schaumberger K, Schernhammer E & Stevens RG. (2006). Cancer and rhythm. *Cancer Causes Control* **17,** 483-487.

Mrosovsky N. (1999). Masking: history, definitions, and measurement. *Chronobiology international* **16,** 415-429.

Nakahata Y, Kaluzova M, Grimaldi B, Sahar S, Hirayama J, Chen D, Guarente LP & Sassone-Corsi P. (2008). The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. *Cell* **134,** 329-340.

Pai-Silva MD, Ueda AK, Resende LA, Pai VD, Alves A, Faleiros AT & De Castro Rodrigues A. (2001). Morphological aspects of rabbit masseter muscle after cervical sympathectomy. *International journal of experimental pathology* **82,** 123-128.

Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS & Hogenesch JB. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* **109,** 307-320.

Pauly JE & Scheving LE. (1967). Circadian rhythms in blood glucose and the effect of different lighting schedules, hypophysectomy, adrenal medullectomy and starvation. *Am J Anat* **120,** 627-636.

Pearen MA, Myers SA, Raichur S, Ryall JG, Lynch GS & Muscat GE. (2008). The orphan nuclear receptor, NOR-1, a target of beta-adrenergic signaling, regulates gene expression that controls oxidative metabolism in skeletal muscle. *Endocrinology* **149,** 2853-2865.

Perreau-Lenz S, Pevet P, Buijs RM & Kalsbeek A. (2004). The biological clock: the bodyguard of temporal homeostasis. *Chronobiology international* **21,** 1-25.

Philipp M & Hein L. (2004). Adrenergic receptor knockout mice: distinct functions of 9 receptor subtypes. *Pharmacology & therapeutics* **101,** 65-74.

Pick J. (1970). *The autonomic nervous system; morphological, comparative, clinical, and surgical aspects*. Lippincott, Philadelphia,.

Pircher P, Chomez P, Yu F, Vennstrom B & Larsson L. (2005). Aberrant expression of myosin isoforms in skeletal muscles from mice lacking the rev-erbAalpha orphan receptor gene. *American journal of physiology* **288,** R482-490.

Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U & Schibler U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110,** 251-260.

Ralph MR, Foster RG, Davis FC & Menaker M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. *Science (New York, NY* **247,** 975-978.

Rattigan S, Appleby GJ, Edwards SJ, McKinstry WJ, Colquhoun EQ, Clark MG & Richter EA. (1986). Alpha-adrenergic receptors in rat skeletal muscle. *Biochemical and biophysical research communications* **136,** 1071-1077.

Reilly DF, Curtis AM, Cheng Y, Westgate EJ, Rudic RD, Paschos G, Morris J, Ouyang M, Thomas SA & FitzGerald GA. (2008). Peripheral circadian clock rhythmicity is retained in the absence of adrenergic signaling. *Arterioscler Thromb Vasc Biol* **28,** 121-126.

Reppert SM & Weaver DR. (2002). Coordination of circadian timing in mammals. *Nature* **418,** 935-941.

Rotto-Percelay DM, Wheeler JG, Osorio FA, Platt KB & Loewy AD. (1992). Transneuronal labeling of spinal interneurons and sympathetic preganglionic neurons after pseudorabies virus injections in the rat medial gastrocnemius muscle. *Brain Res* **574,** 291-306.

Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB & Fitzgerald GA. (2004). BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol* **2,** e377.

Ryall JG, Gregorevic P, Plant DR, Sillence MN & Lynch GS. (2002). Beta 2-agonist fenoterol has greater effects on contractile function of rat skeletal muscles than clenbuterol. *American journal of physiology* **283,** R1386-1394.

Ryall JG, Schertzer JD & Lynch GS. (2007). Attenuation of age-related muscle wasting and weakness in rats after formoterol treatment: therapeutic implications for sarcopenia. *J Gerontol A Biol Sci Med Sci* **62,** 813-823.

Ryall JG, Sillence MN & Lynch GS. (2006). Systemic administration of beta2-adrenoceptor agonists, formoterol and salmeterol, elicit skeletal muscle hypertrophy in rats at micromolar doses. *British journal of pharmacology* **147,** 587-595.

Sato TK, Yamada RG, Ukai H, Baggs JE, Miraglia LJ, Kobayashi TJ, Welsh DK, Kay SA, Ueda HR & Hogenesch JB. (2006). Feedback repression is required for mammalian circadian clock function. *Nature genetics* **38,** 312-319.

Schiaffino S & Reggiani C. (1996). Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiological reviews* **76,** 371-423.

Schiaffino S & Serrano A. (2002). Calcineurin signaling and neural control of skeletal muscle fiber type and size. *Trends Pharmacol Sci* **23,** 569-575.

Serrano AL, Murgia M, Pallafacchina G, Calabria E, Coniglio P, Lomo T & Schiaffino S. (2001). Calcineurin controls nerve activity-dependent specification of slow skeletal muscle fibers but not muscle growth. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 13108-13113.

Shen T, Liu Y, Cseresnyes Z, Hawkins A, Randall WR & Schneider MF. (2006). Activity- and calcineurin-independent nuclear shuttling of NFATc1, but not NFATc3, in adult skeletal muscle fibers. *Molecular biology of the cell* **17,** 1570-1582.

Smith SA, Mitchell JH & Garry MG. (2006). The mammalian exercise pressor reflex in health and disease. *Experimental physiology* **91,** 89-102.

Stephan FK. (2002). The "other" circadian system: food as a Zeitgeber. *Journal of biological rhythms* **17,** 284-292.

Stephan FK, Swann JM & Sisk CL. (1979). Entrainment of circadian rhythms by feeding schedules in rats with suprachiasmatic lesions. *Behavioral and neural biology* **25,** 545-554.

Stokkan KA, Yamazaki S, Tei H, Sakaki Y & Menaker M. (2001). Entrainment of the circadian clock in the liver by feeding. *Science (New York, NY* **291,** 490-493.

Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH & Weitz CJ. (2002). Extensive and divergent circadian gene expression in liver and heart. *Nature* **417,** 78-83.

Stratmann M & Schibler U. (2006). Properties, entrainment, and physiological functions of mammalian peripheral oscillators. *Journal of biological rhythms* **21,** 494-506.

Takahashi JS, Hong HK, Ko CH & McDearmon EL. (2008). The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet* **9,** 764-775.

Terazono H, Mutoh T, Yamaguchi S, Kobayashi M, Akiyama M, Udo R, Ohdo S, Okamura H & Shibata S. (2003). Adrenergic regulation of clock gene expression in mouse liver. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 6795-6800.

Thomas GD & Segal SS. (2004). Neural control of muscle blood flow during exercise. *J Appl Physiol* **97,** 731-738.

Tothova J, Blaauw B, Pallafacchina G, Rudolf R, Argentini C, Reggiani C & Schiaffino S. (2006). NFATc1 nucleocytoplasmic shuttling is controlled by nerve activity in skeletal muscle. *J Cell Sci* **119,** 1604-1611.

Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS & Bass J. (2005). Obesity and metabolic syndrome in circadian Clock mutant mice. *Science (New York, NY* **308,** 1043-1045.

Ueyama T, Krout KE, Nguyen XV, Karpitskiy V, Kollert A, Mettenleiter TC & Loewy AD. (1999). Suprachiasmatic nucleus: a central autonomic clock. *Nat Neurosci* **2,** 1051-1053.

Um JH, Yang S, Yamazaki S, Kang H, Viollet B, Foretz M & Chung JH. (2007). Activation of 5'-AMP-activated kinase with diabetes drug metformin induces casein kinase Iepsilon (CKIepsilon)-dependent degradation of clock protein mPer2. *The Journal of biological chemistry* **282,** 20794-20798.

Vieira E, Nilsson EC, Nerstedt A, Ormestad M, Long YC, Garcia-Roves PM, Zierath JR & Mahlapuu M. (2008). Relationship between AMPK and the transcriptional balance of clock-related genes in skeletal muscle. *Am J Physiol Endocrinol Metab* **295,** E1032-1037.

Vujovic N, Davidson AJ & Menaker M. (2008). Sympathetic input modulates, but does not determine, phase of peripheral circadian oscillators. *American journal of physiology* **295,** R355-360.

Windisch A, Gundersen K, Szabolcs MJ, Gruber H & Lomo T. (1998). Fast to slow transformation of denervated and electrically stimulated rat muscle. *The Journal of physiology* **510 (Pt 2),** 623-632.

Woelfle MA, Ouyang Y, Phanvijhitsiri K & Johnson CH. (2004). The adaptive value of circadian clocks: an experimental assessment in cyanobacteria. *Curr Biol* **14,** 1481-1486.

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC & Spiegelman BM. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98,** 115-124.

Yamamoto T, Nakahata Y, Tanaka M, Yoshida M, Soma H, Shinohara K, Yasuda A, Mamine T & Takumi T. (2005). Acute physical stress elevates mouse period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. *The Journal of biological chemistry* **280,** 42036-42043.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M & Tei H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. *Science (New York, NY* **288,** 682-685.

Yang X, Downes M, Yu RT, Bookout AL, He W, Straume M, Mangelsdorf DJ & Evans RM. (2006). Nuclear receptor expression links the circadian clock to metabolism. *Cell* **126,** 801-810.

Zambon AC, McDearmon EL, Salomonis N, Vranizan KM, Johansen KL, Adey D, Takahashi JS, Schambelan M & Conklin BR. (2003). Time- and exercise-dependent gene regulation in human skeletal muscle. *Genome Biol* **4,** R61.

Zeman RJ, Ludemann R, Easton TG & Etlinger JD. (1988). Slow to fast alterations in skeletal muscle fibers caused by clenbuterol, a beta 2-receptor agonist. *The American journal of physiology* **254,** E726-732.