Norepinephrine regulates mitochondrial biogenesis and function in the hippocampus

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Abstract

Neuronal mitochondria are central to not only maintaining cellular bioenergetics, calcium dynamics and modulation of ROS signalling, but are also critical for specialized functions including synaptic plasticity and neurotransmission. While mitochondria are postulated to have a fundamental role in the functioning of neurons, it's only recently that factors that influence mitochondria in neurons and the central nervous system are being investigated. Here, we identify a critical role of neurotransmitter Norepinephrine in modulating mitochondria in rodent hippocampal cells. Norepinephrine increases mitochondrial biogenesis, enhances the expression of regulators of mitochondrial biogenesis, regulates ATP synthesis and influences mitochondrial function. Increasing Norepinephrine content at the synapse via treatment with selective norepinephrine reuptake inhibitors, also evoked robust increases in mitochondrial biogenesis and ATP content. These effects of Norepinephrine appear to be mediated by the β adrenergic receptor subtypes and involve a critical role of the master modulator of mitochondrial biogenesis PGC1a. These findings identify a novel and exciting role for Norepinephrine in impacting mitochondrial turnover and biogenesis in the hippocampus.

Introduction

Norepinephrine has been implicated in regulating bioenergetics in various cell types, regulating both the generation and function of mitochondria, the powerhouse of the cell, as well as influencing the availability of ATP, the energy currency of the cell [1]. Neurons are the longest-lived of all cell types and use a lot of energy, not simply for their baseline activity but also to facilitate rapid shifts in neuronal activity that are essential for the spectrum of brain functions. Neurons consume more than twenty per cent of the energy generated by food consumption. The ability to generate new mitochondria, turnover old mitochondria and produce the energy currency of ATP is vital for neurons [14,19]. Even subtle vicissitudes in mitochondrial function are known to compromise overall neuronal health and status, which places mitochondria as a central organelle to be targeted for therapeutics that boost neuronal viability. The central question that we wanted to address is how Norepinephrine can influence mitochondrial numbers, function and bioenergetics within important brain regions, in particular in the hippocampus that is so central to the regulation of learning, memory and mood [3,6,13].

We have looked at the role of Norepinephrine in the regulation of mitochondrial biogenesis and function. Using a wide array of approaches and model systems, we have observed that exogenous Norepinephrine increases mitochondrial number and function in hippocampal neurons. These effects are rapid and arise through Norepinephrine switching on a genetic program that drives mitochondrial production in hippocampal neurons. Experiments using drugs that are routinely administered clinically for the management of ADHD and that are known to elevate Norepinephrine levels in the brain indicate robust increases in mitochondrial number and function in the hippocampus. We have systematically dissected the contribution of the Norepinephrine receptors and molecular players in hippocampal neurons that mediate these effects. We find that the effects may be regulated by β adrenergic receptor-mediated recruitment of PGC-1α. Our results put forward an important role of Norepinephrine in the modulation of mitochondrial biogenesis and function in hippocampal neurons.

Materials and Methods

Animals

All the in-vitro experiments were done on primary hippocampal neurons using embryos derived at E 18.5 from the time pregnant Sprague Dawley dams bred at the Tata Institute of Fundamental Research (TIFR) animal facility. For in vivo experiments, 2-3 months old Sprague Dawley male rats and C57 BL6 male mice were used.

Primary Cell culture

Dams were euthanized with CO2 and embryos were harvested in ice-cold Hanks' balanced salt solution (HBSS). Hippocampi were dissected in minimum essential medium, and then enzymatically dissociated in 0.05% trypsin/EDTA for 15 min. The tissue was then triturated to obtain a single cell suspension in Neurobasal medium containing 2% B27, 0.5mM L-glutamine and Penicillin-Streptomycin. Cells were seeded in plates coated with 0.1 mg/ml poly-D-lysine at a density of 1 million cells per well 9.6 cm sq. Cells were allowed to differentiate for a minimum of 6 days before treatments.

Drug treatment paradigms

For looking at changes in gene expression, hippocampal neurons were treated with Norepinephrine (0.1, 1 uM) for 30mins. For changes in protein expression, hippocampal neurons were treated with NE (0.1, 1 uM) for 4hrs. For changes in mitochondrial DNA content and ATP production, hippocampal neurons were treated with Norepinephrine (0.1, 1 uM) for 8hrs. To block the β adrenergic receptors, hippocampal neurons were treated with NE (0.1 uM) for 8hrs for ATP production in the presence of β adrenergic antagonist, Propranolol (10uM). For experiments with the β adrenergic agonist, hippocampal neurons were treated with Isoproterenol (0.1, 1 uM) for 30mins for gene expression and with Isoproterenol (0.1uM) for 8hrs for mitochondrial DNA content and ATP production. To mimic cyclic AMP signalling, Forskolin (0.1, 1 uM) for 30mins for gene expression and 8hrs for ATP production. Mouse hippocampal cultures from PGC1αco/co were treated with NE (0.1 uM) for 8hrs for mitochondrial DNA content and ATP production.

For in vivo experiments, Sprague-Dawley rats received intraperitoneal administration of Atomoxetine (3 mg/kg) or vehicle (0.9 % saline) once daily for one or three days and were sacrificed two hours after the last treatment. For blocking β adrenergic receptors, Propranolol (0.5g/l) was given in drinking water. For intrahippocampal delivery of Norepinephrine, vehicle (Phosphate buffered saline) or NE (100 nM) were infused in the dorsal hippocampus via stereotactic delivery through an osmotic minipump (Alzet Model 2001) for 3 days. C57BL6 mice received intraperitoneal administration of Isoproterenol (2 mg/kg) or vehicle (0.9 % saline) once daily for one or three days and were sacrificed 4 hours after the last treatment.

Quantitative real time polymerase chain reaction

RNA was extracted from hippocampal neurons using Trizol® (Ambion) method. 1 ug of RNA per sample was reverse transcribed to cDNA using random hexamers and the PrimeScript reverse transcription kit (Takara, Japan). Quantitative real time PCR was done using gene-specific primers and cDNA was amplified in the Biorad real time PCR system using KAPA SYBR® FAST Universal 2X qPCR Master Mix (Kapa Biosystems). Gene expression levels were normalized to the 18S ribosomal RNA per sample, and the relative expression levels between control and treated samples were computed by the Ct method. Data are represented as fold change ± SEM as compared to control.

Mitochondrial DNA content

Mitochondrial DNA (mtDNA) content was compared in control versus treated hippocampal neurons by quantitative real time PCR. Genomic DNA was extracted from cells using the commercially available PureLink® Genomic DNA Mini Kit (Invitrogen). Cytochrome B - a mitochondrial genome encoded gene were normalized to levels of a nuclear-encoded gene cytochrome C. Relative mitochondrial DNA levels between groups were quantified by the Ct method.

Cellular ATP

Cellular ATP was measured as described previously[9]. Briefly, hippocampal neurons or tissue were lysed in boiling water and the lysate was centrifuged at 14,000 rpm for 20 min at 4°C. The ATP levels in the supernatant were measured using the ATP bioluminescent assay kit (Sigma-Aldrich. The light emitted is proportional to the ATP consumed in the reaction, and was measured using a luminometer (Berthold Technologies, Germany). ATP readings were normalized to the protein content of each sample, estimated using a BCA protein assay kit (Sigma-Aldrich).

Western blot analysis

Hippocampal neuron cultures were lysed in ice-cold Radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl), with added protease and phosphatase inhibitors (Roche Applied Science). Lysates were centrifuged at 13000 rpm for 20 min and the supernatant was collected. Protein estimation was completed using the QuantiPro BCA (Bicinchoninic Acid) assay kit (Sigma-Aldrich) and lysates were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto polyvinylidene fluoride (PVDF, Merck Millipore, MA, USA) membranes, blocked in 5% BSA in Tris Buffered Saline-Tween (0.1%) and probed with primary antibodies in 5% BSA (bovine serum albumin) overnight at 4°C.

Primary antibodies included rabbit anti-VDAC (1:1000, Abcam), rabbit anti-BDNF (1:1000, Abcam), and rabbit anti-actin (1:5000, Abclonal). Blots were washed and incubated with goat anti-rabbit IgGperoxidase labelled (1:10000, Alclonal) secondary antibodies for 2 hours at room temperature. Signal was detected using a chemiluminescence kit (Thermo Fisher Scientific), and bands were visualized on the GE Amersham Imager 600. Actin was used as loading control for normalization. The relative density of bands was measured using ImageJ.

Osmotic Minipump Surgery

For intrahippocampal delivery of Norepinephrine, vehicle (Phosphate buffered saline) or NE (100 nM) were infused in the dorsal hippocampus (relative to bregma: AP: -3.6mm, ML: +2.00mm, DV: -2.8mm) of Sprague Dawley rats (3–4-month-old) via stereotactic delivery through an osmotic minipump (Alzet Model 2001) for 3 days. Pumps were attached to the cannula and inserted in a subcutaneous pocket made at the back of the animal, and animals were closely monitored for post-surgical recovery.

Results

Norepinephrine regulates mitochondrial biogenesis and function

We examined the effect of treating hippocampal neurons with monoamine Norepinephrine on the expression of genes regulating mitochondrial biogenesis. 0.1 and 1uM NE treatment for 30 minutes evoked upregulation of Sirt1, Ppargc1a, Tfam, ATP5a, CytC, and Nrf1 (Figure 1 A – G). 0.1 and 1uM NE treatment for 4 hours also upregulated protein level expression of mitochondrial outer

membrane marker VDAC and neurotrophic factor BDNF (Figure 1 H –J). We next looked at the effect of 0.1 and 1uM NE treatment at 8hrs which showed an increase in mt DNA content and ATP production (Figure 1 K –M).

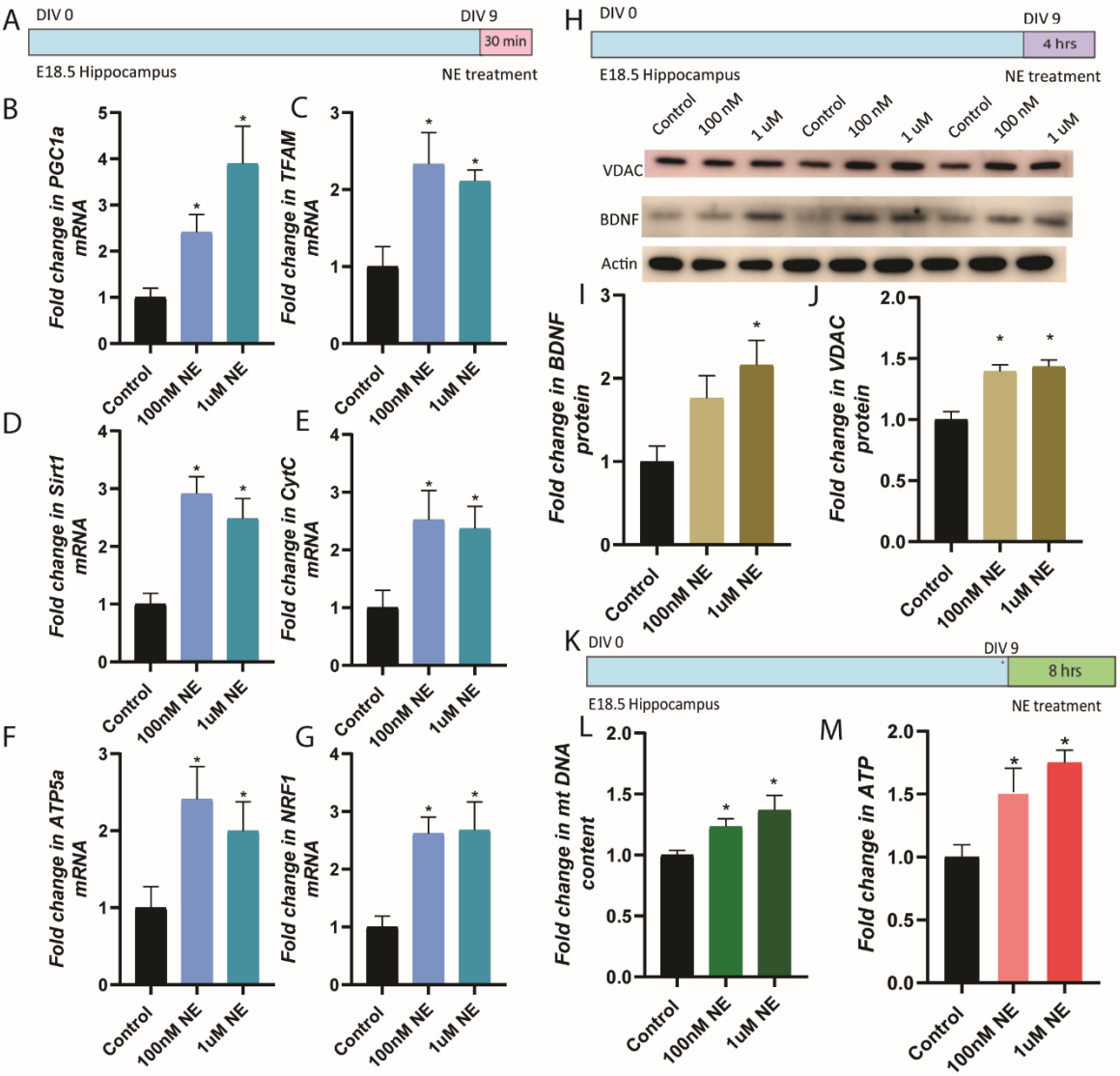


Figure 1: Mitochondrial biogenesis and function are regulated by Norepinephrine in hippocampal neurons. (A) Shown is a schematic representation of Norepinephrine treatment paradigm for gene expression. (B-G) Quantitative PCR (qPCR) analysis for mRNA expression of major modulators of mitochondrial biogenesis Ppargc1a (B), Tfam (C), Sirt1(D), CytC (E), Atp5a(F) and NRF1 (G) are shown as fold change of control ± SEM (representative results from n = 4 per treatment group/N = 3). (H) Shown is a schematic representation of Norepinephrine treatment paradigm for protein expression. (I, J) Quantitative densitometric analysis of VDAC (I) and BDNF (J) immunoblots. Results are expressed as fold change of control ± SEM (Results from n = 3 per treatment group/N = 2, (K) Shown is a schematic representation of Norepinephrine treatment paradigm for mitochondrial DNA content and ATP production. (L) qPCR analysis for mtDNA levels is shown as relative mtDNA content ± SEM (representative results from n = 4 per treatment group/N = 2). (M) Quantitation of ATP levels represented as fold change of control ± SEM (representative results from n = 4 per treatment group/N = 3). *P < 0.05 (compared with control, one-way ANOVA, Tukey's post hoc test).

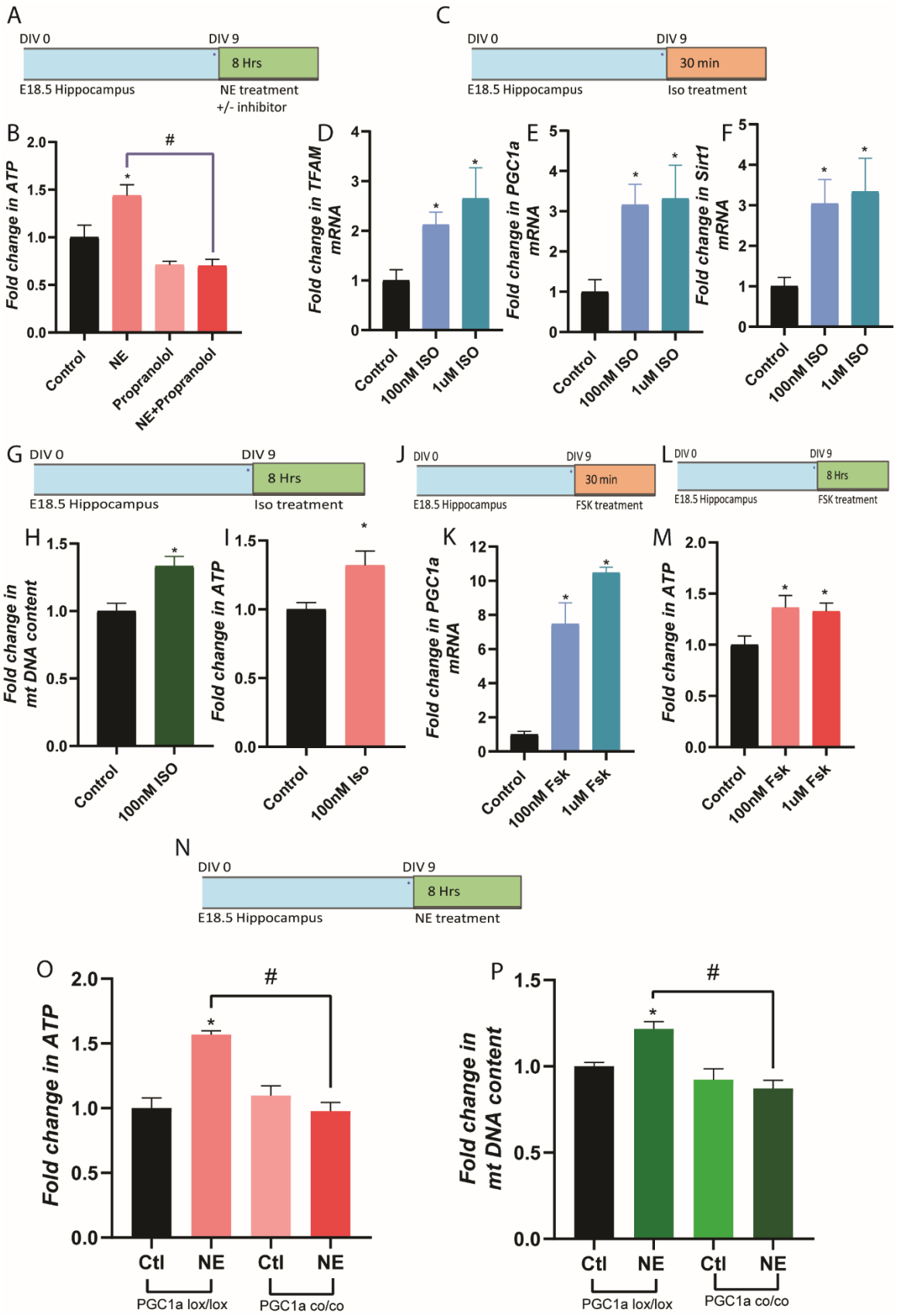
Norepinephrine regulates mitochondrial biogenesis and function via the β adrenergic receptors and PGC1a pathway

β adrenergic receptors have been implicated in mediating mitochondrial effects of Norepinephrine in other tissues including muscle and heart [16,17,24]. To investigate if the β adrenergic class of adrenergic receptors mediated Norepinephrine's role in modulating mitochondrial biogenesis in hippocampal neurons, we decided to block β adrenergic receptors using propranolol, a broad β adrenergic receptor antagonist. 10 uM Propranolol significantly reduced the effect of norepinephrine on ATP production (Figure 2 A, B). Further, β adrenergic agonist Isoproterenol was also seen to mimic the effects of Norepinephrine, including an increase in expression of genes regulating mitochondrial biogenesis, mitochondrial DNA content and ATP production (Figure 2 C-I). Since β adrenergic receptors function by the Gs signalling, we used forskolin to see if it mimics the effects of Norepinephrine treatment. Forskolin also showed an increase similar to Norepinephrine in Ppgarc1 gene transcription and ATP content (Figure 2 J-M).

The β-adrenergic/cAMP pathway is known to activate PGC1a gene transcription [25]. PGC1a is highly inducible in response to physiologic conditions that demand increased mitochondrial energy production [20]. We investigated the possible involvement of PGC1a in the actions of 5-HT on mitochondria by treating hippocampal neurons from neurons derived from PGC1a lox/lox embryos and invitro transduced with adeno-associated virus (AAV) Cre− or AAV Cre+ to yield control (PGC1a lox/lox) or PGC1a loss-of-function (PGC1a co/co) neurons, respectively. Norepinephrine treatment did not alter the mitochondrial DNA content or the ATP content in the absence of PGC1a loss of function (PGC1a co/co), in contrast to the robust upregulation noted in control PGC1a lox/lox hippocampal neuron (Figure 2 N-P).

Figure 2: Norepinephrine modulates mitochondrial biogenesis and function via the β adrenergic receptors. (A) Shown is a schematic representation of Propranolol and Norepinephrine treatment paradigm for ATP production. (B) Quantitation of ATP levels in hippocampal neurons treated with Norepinephrine in the presence or absence of Propranolol are expressed as fold change of control ± SEM (representative results from n = 4 per treatment group/N = 2) (C) Shown is a schematic representation of Isoproterenol treatment paradigm for gene expression. (D-F) Quantitative PCR (qPCR) analysis for mRNA expression of major modulators of mitochondrial biogenesis Tfam (D), Ppargc1a (E), and Sirt1(F) are shown as fold change of control ± SEM (representative results from n = 4 per treatment group/N = 3). (G) Shown is a schematic representation of Isoproterenol treatment paradigm for mitochondrial DNA content and ATP production. (H) qPCR analysis for mtDNA levels is shown as relative mtDNA content ± SEM (representative results from n = 4 per treatment group/N = 2). (I) Quantitation of ATP levels represented as fold change of control ± SEM (representative results from n = 4 per treatment group/N = 3). (J) Shown is a schematic representation of Forskolin treatment paradigm for gene expression. (K) Quantitative PCR (qPCR) analysis for mRNA expression of Ppargc1a are shown as fold change of control ± SEM (representative results from n = 4 per treatment group/N = 2). (L) Shown is a schematic representation of Forskolin treatment paradigm for ATP production. (M) Quantitation of ATP levels represented as fold change of control ± SEM (representative results from n = 4 per treatment group/N = 1). (N) Shown is a schematic representation of Norepinephrine treatment paradigm for mitochondrial DNA content and ATP production in PGC1a $^{co/co}$ hippocampal culture. (O) Quantitation of ATP levels represented as fold change of control ± SEM (representative results from n = 4 per treatment group/N = 2). (P) qPCR analysis for mtDNA levels is shown as relative mtDNA content ± SEM (representative results from n = 4 per treatment group/N = 1). *P < 0.05 (compared with control, one-way ANOVA, Tukey's post hoc

test). #P < 0.05 (compared with Norepinephrine treated group); two-way ANOVA, Tukey's post hoc test.



In Vivo Regulation of hippocampal mitochondrial biogenesis by Norepinephrine.

We next sought to investigate the effects of elevating NE in vivo on mitochondrial biogenesis in the hippocampus. We used Atomoxetine, a selective Norepinephrine reuptake inhibitor to increase Norepinephrine content in the hippocampus [5]. An acute dosage (sacrifice 2 hr post-treatment) with 3mg/kg Atomoxetine triggered a significant increase in gene expression of Ppargc1a, Sirt1, Tfam, and Cycs in the rat hippocampus (Figure 3 A-E). Subchronic treatment (3 days) treatment with Atomoxetine showed an increase in gene expression of Ppargc1a, Sirt1, Nrf1, Tfam, and ATP5a (Figure 3 F-L).

To address if NE functions via β adrenergic receptors, we used β adrenergic antagonist Propranolol in drinking water (0.5g/l) to investigate if blocking β adrenergic receptors inhibits the effect of Atomoxetine on the mitochondrial biogenesis in the rat hippocampus. While three-day Atomoxetine administration resulted in a significant induction of mtDNA levels and ATP content over the control animals, this was lost in animals treated with Propranolol (Figure 3 M-O).

Next, to investigate the effects of direct upregulation of Norepinephrine in the hippocampus, we directly delivered NE (100 nM, 3 d) into the dorsal hippocampus of Sprague–Dawley rats using Alzet osmotic minipumps. Intrahippocampal infusion of NE also resulted in significant increases in mitochondrial DNA levels (Figure 3 P, Q).

Further, in C57BL6N mice, acute treatment with β adrenergic agonist 2mg/kg Isoproterenol (sacrifice 4 hr post-treatment) caused a significant increase in gene expression of Ppargc1a in the rat hippocampus (Figure 3 R, S). Subchronic treatment (3 days) with Isoproterenol gave increased mitochondrial DNA content (Figure 3 T, U).

Figure 3: In Vivo Regulation of hippocampal mitochondrial biogenesis by Norepinephrine. (A) Shown is a schematic representation of acute Atomoxetine treatment paradigm for gene expression. (B-E) Quantitative PCR (qPCR) analysis for mRNA expression of major modulators of mitochondrial biogenesis Ppargc1a (B), Sirt1(C), CytC (D), and Tfam (E)are shown as fold change of control ± SEM (n = 8). (F) Shown is a schematic representation of sub chronic Atomoxetine treatment paradigm for gene expression. (G-L) Quantitative PCR (qPCR) analysis for mRNA expression of major modulators of mitochondrial biogenesis Ppargc1a (G), Sirt1(H), Tfam (I), ATP5b (J), NRF1 (K), and CytC (L) are shown as fold change of control ± SEM (n = 7-8). (M) Shown is a schematic representation of Propranolol and Atomoxetine treatment paradigm for mitochondrial DNA content and ATP production. (N) qPCR analysis for mtDNA levels is shown as relative mtDNA content ± SEM (n = 8). (O) Quantitation of ATP levels represented as fold change of control ± SEM (n = 8). (P) Shown is a schematic representation of Propranolol and Atomoxetine treatment paradigm for mitochondrial DNA content. (Q) qPCR analysis for mtDNA levels is shown as relative mtDNA content ± SEM (n = 7). (R) Shown is a schematic representation of Isoproterenol treatment paradigm for gene expression. (S) Quantitative PCR (qPCR) analysis for mRNA expression of Ppargc1a are shown as fold change of control ± SEM (n=8). (T) Shown is a schematic representation of Isoproterenol treatment paradigm for mitochondrial DNA content. (U) qPCR analysis for mtDNA levels is shown as relative mtDNA content ± SEM (n = 8). *P < 0.05 (compared with control, one-way ANOVA, Tukey's post hoc test). #P < 0.05 (compared with Atomoxetine treated group); two-way ANOVA, Tukey's post hoc test.

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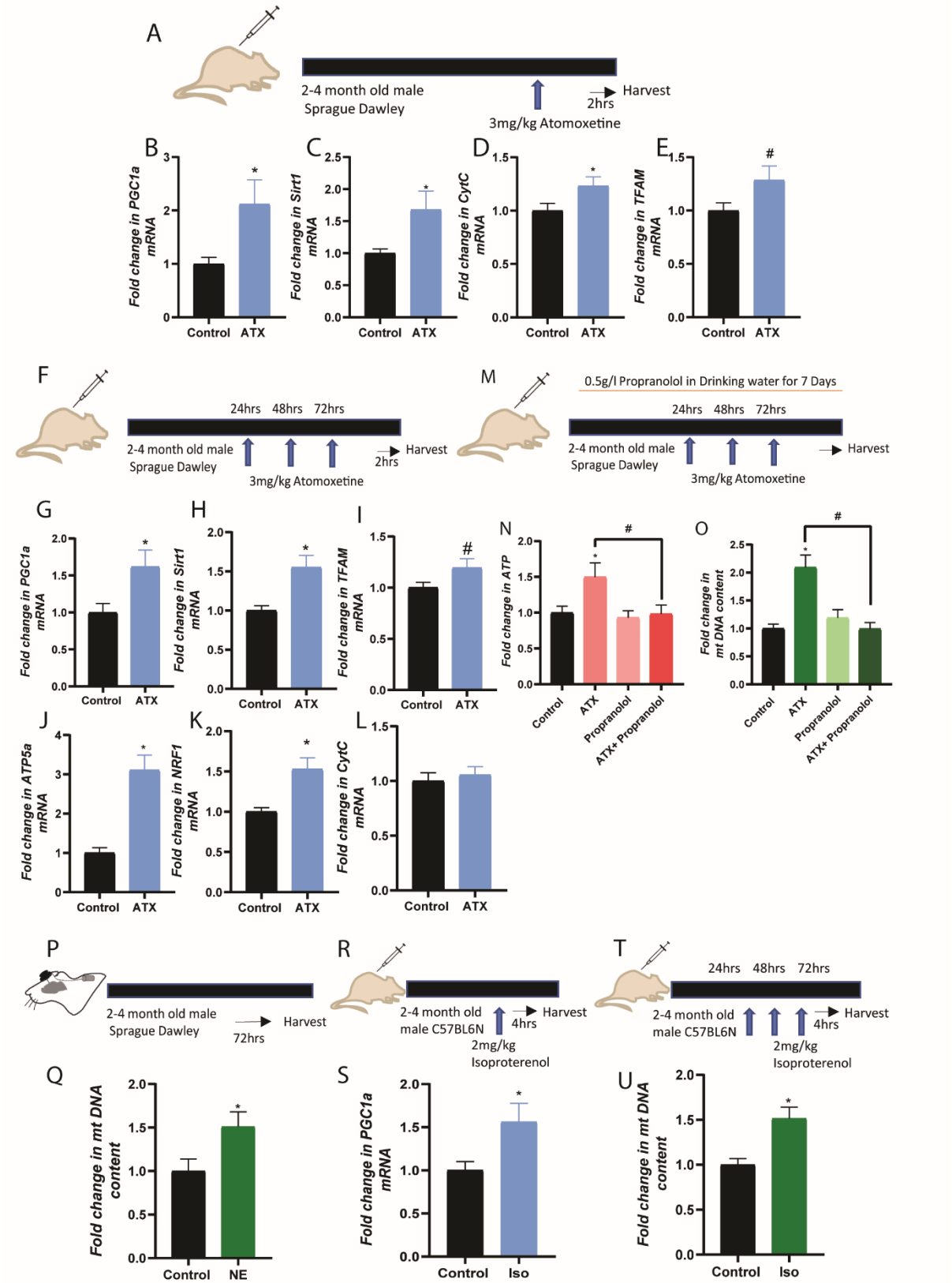

Discussion

Our findings show that Norepinephrine regulates both mitochondrial biogenesis and function in the hippocampus and cultured hippocampal neurons, via the β adrenergic receptors. Physiologically

relevant doses of Norepinephrine show an increase in transcription of Ppgarc1 and TFAM which are critical genes to driving mitochondrial biogenesis, mitochondrial DNA content and ATP production. Prior reports indicated that specific Norepinephrine receptors can influence mitochondrial biogenesis in nonneuronal cells, such as renal proximal tubular cells, adipose tissue and cardiomyocytes. Thus far, few studies have examined the direct influence of Norepinephrine on mitochondria in neurons. Direct application of Norepinephrine on cortical neurons has been shown to not affect mitochondrial DNA content or ATP production[9].

A recent study has demonstrated that β adrenergic receptor 2 stimulation rescues mitochondrial biogenesis in a model of traumatic brain injury [12]. β adrenergic stimulation is also shown to increase mitochondrial biogenesis in mice kidney, muscle and heart [2,8,16,17,22,24]. We demonstrate that blocking the β adrenergic receptors using Propranolol blocked the effects of Norepinephrine on ATP levels. Also, broad β adrenergic receptor agonist Isoproterenol shows effects similar to Norepinephrine on mitochondrial DNA content and ATP production. Mimicking the stimulation of the β adrenergic receptors by the cAMP modulator, Forskolin also enhances the transcription of Ppgarc1 and ATP production. As β adrenergic receptors are coupled to the Gs pathway, this increase in mitochondrial biogenesis may be driven by the cAMP-CREB-PGC1a pathway. This pathway has been implicated in mitochondrial biogenesis in several tissues including glioblastoma cells and hepatocytes [7,25]. PGC1a has emerged as a critical metabolic node and as a master modulator of mitochondrial biogenesis in several cell types, including neurons [4,10]. We show that PGC1a is essential for the mitochondrial effects of Norepinephrine on hippocampal neurons.

Recently several papers have talked about the role of neurotransmitters in regulating mitochondrial biogenesis and function in the brain. Dopamine receptors have been shown to regulate mitochondrial respiration in nucleus accumbens [18]. Serotonin has been shown to affect mitochondrial biogenesis via the 5HT-2A receptor and Sirt1/PGC1a pathway in the cortical neurons[9]. Melatonin and N-acetyl serotonin are also known to exert neuroprotective effects which may involve inhibition of mitochondrial death pathways [11,26]. This suggests that different monoamines can have circuit-specific influences on mitochondria.

To investigate the role of norepinephrine in modulating the mitochondria in the adult rodent hippocampus, we have used selective norepinephrine reuptake inhibitor Atomoxetine to elevate extracellular norepinephrine levels. Atomoxetine has been shown to rapidly and persistently increase norepinephrine in several brain regions including the prefrontal cortex, occipital cortex, lateral hypothalamus, dorsal hippocampus, and cerebellum [5]. Atomoxetine is most widely used for the treatment of attention deficit hyperactivity disorder (ADHD) in both children and adults. Atomoxetine has been shown to rescue synaptic plasticity by re-establishing long term potentiation in rodent models of ADHD, which may underlie improvement of ADHD symptoms [21]. It is interesting to note that synaptic plasticity and long-term potentiation are also modulated by mitochondrial health and function [19]. Also, a few studies have reported modulated mitochondrial biogenesis and function and PGC1a regulation by Atomoxetine in SH-SY5Y cells and skeletal muscle [15,23]. We show that Atomoxetine directly increases mitochondrial DNA content, Ppargc1a and Sirt1 gene expression, and ATP levels in the rodent hippocampus.

In conclusion, our results show that Norepinephrine can increase mitochondrial biogenesis and function in hippocampal neurons, possibly via a β adrenergic receptor-dependent recruitment of the cAMP-PGC1a axis.

Statistics

The data was subjected to an ordinary one-way ANOVA using GraphPad Prism (Graphpad Software Inc., USA). For experiments with blockers and knockout lines, the data was subjected to a Two-way ANOVA using GraphPad Prism (Graphpad Software Inc., USA). Data are expressed as mean ± standard error of the mean (S.E.M) and statistical significance was set at $p < 0.05$.

Statistical outliers were removed when they were more than three standard deviations away from the mean and were outliers by Grubb's test.

Significance to humankind

Neurodevelopmental and neuropsychiatric disorders profoundly impact the quality of health and life of affected people and are responsible for substantial economic burden. In India, 197 million people were reported to have neurodevelopmental and neuropsychiatric disorders of varying severity in 2017, which is approximately one in seven people. Amongst these disorders is attention deficit hyperactivity disorder (ADHD), with symptoms including hyperactivity, irritability and aggression, with inattentiveness and forgetfulness. Several recent studies have reported the prevalence of ADHD from 1.6 per cent to 17.9 per cent in adolescents in India. The burgeoning load of neurodevelopmental and neuropsychiatric disorders is substantial for both developed and developing countries, and with countries that have a large younger population, ADHD has critical long-term implications. It is vital to understand the neurobiological mechanisms that drive both the genesis of these disorders, as well as to identify the effective therapies for the wide spectrum of diseases subsumed under neurodevelopmental and neuropsychiatric disorders. In this regard, it is of substantial interest that the neurotransmitter Norepinephrine, which is a major modulator of attention, learning, memory and mood states, is implicated both in the symptoms and treatment of these diseases. Several drugs that treat ADHD, as well as antidepressant treatments used for mood disorders, capitalize on approaches to increase Norepinephrine levels in the brain. This research has the potential to be path-breaking by showing that a neurotransmitter like Norepinephrine, which thus far is implicated in the modulation of attention, learning, memory and mood, also plays a critical cellular role in influencing the energy-generating organelle, mitochondria and the capacity of a neuron to dynamically modulate its bioenergetics. This opens up central questions for further enquiry, raising the possibility of novel targets to treat neurodevelopmental and neuropsychiatric disorders.

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