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태내 스트레스에 의한 일주기 리듬 불균형과 대사장애

Effect of maternal stress on circadian rhythm and metabolism

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서울대학교 대학원

생명과학부

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윤 성 식의 이학석사 학위논문을 인준함

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Effect of maternal stress on circadian rhythm and metabolism

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December, 2012

Date approved

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CONTENTS

ABSTRACT

It is well established that maternal stress causes defective functions in several important brain regions such as hypothalamus, hippocampus and amygdala. However, the effect of maternal stress on the suprachiasmatic nucleus (SCN) which acts as a central pacemaker driving circadian rhythm remains largely unknown. In the present study, I focused on the question whether stress in pregnant mice could influence the circadian clockwork in the SCN of their offspring and its physiological relevance. SCN slices from 1 week-old transgenic mice maintained rhythmic PER1::LUC expression for more than a week, and maternal stress caused shortened periodicity and reduced amplitude, and even loss of rhythm in some cases. However, in SCN slices from 5 week-old mice, altered period and arrhythmicity were recovered as similar to those of adult mice while the amplitude of PER1::LUC was still lower than the control group. In addition, mRNA profiles of several molecular clock genes in liver of prenatally stressed adult mice were affected. In accordance with the liver clock, rhythmic profiles of metabolic genes involved in glucose metabolism were altered in maternally stressed mice. However, although liver glycogen contents were slightly decreased, plasma glucose levels were not changed, suggesting that maternal stress cannot disrupt glucose homeostasis in normal feeding

condition. Taken together, these findings suggest that maternally stressed mice can adjust to their environment after birth, likely for overcoming the effect of prenatal stress on circadian timing system

Key words: Maternal stress, Suprachiasmatic nucleus (SCN), Circadian rhythm, Metabolic dysregulation.

INTRODUCTION

The environment in early life affects the life of offspring from fetus to adult, which is known as “programming effects” (Seckl, 2008). For example, it is a common notion that pups from mothers stressed in pregnancy show low birth weight, correlated with pathological symptoms such as cardiovascular diseases and metabolic disorders in adulthood (Nathanielsz, 1999; Welberg and scekl, 2001). Previous studies have investigated the long-lasting influences of maternal stress. For example, maternally stressed rat exhibited lower sensitivity of the negative feedback of the hypothalamus-pituitary-adrenal (HPA) axis in adolescent and 16 month-old adult. Also, hippocampal MR and GR receptor showed decreased binding capacity in adult males (Darnaudery and Maccari, 2008)

Glucocorticoid (GC) is believed as a critical hormone which can mediate the effect of prenatal stress. Under stressful circumstances, HPA axis is activated to respond and to cope with stress by regulating the synthesis and secretion of GC. As the final regulator of stress-responsive neuroendocrine axis, GC exerts widespread actions throughout the body such as modulating metabolites, regulating immune-inflammatory responses, and controlling emotions. (Chrousos and Kino, 2007; Sapolsky et al., 2000; Munck and Náray-Fejes-Tóth, 1992). In addition, under

undisturbed states, GC exhibits a rhythmic fashion in secretion throughout a day so this rhythm is able to respond to stress and make GC-dependent genes also have a pulsatile expression (Lightman et al., 2008; Lightman and Conway-Campbell, 2010).

Circadian rhythm is biological oscillation with a period of approximately 24 hour. This rhythm is not simply a response to daily light-dark cycle; rather, it is regulated by an internal timekeeping system, called 'biological clock' (Dunlap, 1999; Reppert, 1998). This timekeeping system is operated and maintained by cell-autonomous molecular clockwork, referred as 'biological clock machinery'. In the molecular clock, Clock and Bmal1 form a heterodimer and activate the transcription of their targets as a positive regulator by binding to E-box element of downstream genes such as *Periods* (PERs: PER1, PER2 and PER3) and *Cryptochromes* (CRYs: CRY1 and CRY2), the negative regulators (King et al., 1997; Gekakis et al., 1998; Bunger et al., 2000). These PERs and CRYs repress the E-box mediated transcriptional activity of the Clock-Bmal1 heterodimer; therefore they inhibit their own gene expression, and this called the core feedback loop (Kume et al., 1999). In the meantime of core feedback regulation, RORα and Rev-erbα form the auxiliary feedback loop and control the concentration of Bmal1. RORα and Rev-erbα competitively bind to ROR-responsive elements (RRE), and RORα induces Bmal1 transcription whereas Rev-erbα strongly suppresses it (Guillaumond et al., 2005). These

transcriptional/translational feedback loops make the 24 hour period rhythm.

Mammalian circadian system is organized hierarchically. The hypothalamic SCN plays an important role as a master pacemaker in circadian rhythm, receiving information on the day/night cycle and driving virtually all of the diurnal or circadian metabolism, physiology and behavior. Recently, it is revealed that most cells in other tissues and organs harbor their own molecular oscillator with a similar molecular makeup of that in the SCN pacemaker neurons, which are referred to as ‘peripheral or local clocks’. Therefore, SCN can synchronize and harmonize these body clocks, to adjust it to environmental or endogenous clock time (Stratmann and Schibler, 2006).

There are some links between maternal stress and circadian clock. Rhythmic GC signaling has roles in synchronization of body rhythm, such as locomotor activities and clock gene expression in the body (Balsalobre et al., 2000; Chrousos and Kino, 2007). Therefore, it is possible that altered circadian GC signaling in stressed mother may influence the circadian timing system of fetus. Several lines of studies also indicate that maternal stress in rodent causes advanced locomotor activities and GC diurnal rhythm in rodent offspring (Koehl et al., 1997; Maccari et al., 1997).

In addition, there are similarities of metabolic pathophysiology caused by maternal stress or defective clock function. Animal models whose circadian clock components were genetically ablated frequently show

abnormal carbohydrate and lipid metabolism. For example, $Clock^{\Delta 19/\Delta 19}$ mutant mice and pancreas-specific Bmal1 mutant mice exhibit β-cell defect and thereby result in hyperglycemia and hypoinsulinemia (Marcheva et al., 2010). Also, knockout mice of another component of the core loop, *Cryptochrome*, show abnormal glucose homeostasis (Lamia et al., 2011). In other studies, liver-specific knockout of Rev-erbα and β, which form the auxiliary loop, results in dysregulation of carbohydrate and lipid metabolism (Cho et al., 2012). These studies obviously inform the important roles of tissue-specific peripheral clockwork in the regulation of circadian physiology and behavior, particularly in metabolism. There are many evidences that either maternal stress or exposure to high levels of glucocorticoid also induces metabolic unbalances especially including glucose metabolism, similar to consequences of clock gene mutants. Prenatal stress causes hyperglycemia, glucose intolerance, and decreased basal leptin levels (Lesage et al., 2004). These phenomena are due to altered gene expression related to glucose or lipid metabolism. In the adult rat liver, gene transcription of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in gluconeogenesis, is increased by exposure to dexamethasone, a synthetic GC agonist, in utero (Nyirenda et al., 1998). Also, in the placenta of prenatally stressed rat, glucose transporter1 (GLUT1) expression was decreased, whereas GLUT3 and GLUT4 were slightly increased (Mairesse et al., 2007). But the evidences did not sufficiently

demonstrate what exactly cause these metabolic dysregulations. Thus these several features between clock gene deletion models and maternal stress model imply that metabolic disorders in maternal stress model could be caused by disrupted circadian rhythm because of stress in utero.

Previous studies have recently shown that maternal stress has prolonged effect on susceptibility to chronic stress (Chung et al., 2005), hippocampus-dependent spatial learning and memory (Son et al., 2006), dopamine-dependent hyperactivity (Son et al., 2007), and amygdale-dependent fear memory consolidation (Lee et al., 2011). These studies imply that prenatal stress can have profound effects on the functions of various brain regions in adult offspring. However, the impact of maternal stress on the SCN and circadian rhythm remains largely unknown. Thus, I aimed to investigate the effect of maternal stress on the circadian rhythm, and to clarify the metabolic dysregulations which are shown in the maternally stressed mice are the consequences of disrupted circadian rhythm.

MATERIALS AND METHODS

Animal care and handling. ICR mice, obtained from the Laboratory Animal Center at Seoul National University, were used in all of the experiments and kept in temperature-controlled (22-23^{o}C) quarters under a 12h light-dark (LD) photoperiod (light on at 8:00 A.M.). Standard mouse chow and water were available *ad libitum*. For dark-dark (DD) conditions, mice were kept in constant darkness for the indicated duration from the light-off time. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University.

Maternal stress procedure. The maternal stress procedure was performed as described previously (Chung et al., 2005; Son et al., 2006,2007; Choe et al., 2011; Lee et al., 2011). Briefly, pregnant ICR mice were prepared by mating at the age of 6-7 weeks with adult males. Pregnant mice in the stress group were placed individually in a restrainer (a transparent plastic cylinder, 3 cm in diameter and 9 cm long) daily for 6 hours (10:00 a.m. to 4:00 p.m.) from 8.5 days post coitum (dpc) to 18.5 or 19.5 dpc (the day before parturition). Control pregnant mice remained undisturbed. After weaning on postnatal day 21, the pups born from stressed mother (STR) were reared in an environment identical to that of

the controls (CTL). The STR and CTL groups were separately housed, but four to six mice from different litter were randomly assigned to a cage to exclude possible litter effects. Male offspring at 10~12 weeks of age were used in all experiments, except for *ex vivo* tissue explant culture. 1 week-old and 5 week-old PER1::Luc transgenic mice were used in *ex vivo* experiments..

Preparation of plasma and tissues. All mice were sacrificed at the indicated time points with cervical dislocation followed by decapitation for collecting trunk blood. Tissues were isolated on ice and quickly frozen in liquid nitrogen. Frozen tissues and EDTA-plasma were stored at -70℃ until assays.

RNA isolation and RT-PCR. RNA analyses were performed as described previously with modifications (Chung et al., 2005). Mouse tissues were rapidly removed, frozen in liquid nitrogen, and store at -70 ^{o}C until use. Total RNA was isolated by the single-step acid guanidinium thiocyanate-phenol-chloroform method. For RT-PCR, 500 ng of each RNA sample was reverse-transcribed with MMLV reverse transcriptase (Promega, Madison, WI). Then aliquots of the cDNA were subjected to conventional PCR or quantitative real-time PCR in the presence of SYBR Green I (Sigma, St. Louis, MO). Gene expression levels were normalized with Glyceraldehyde

3-phosphate dehydrogenase (GAPDH). Primer sequences used for real-time RT-PCR are shown in Table 1 for circadian clock genes and Table 2 for metabolic genes.

Measurement of metabolic parameters. EDTA-plasma was prepared from trunk blood as described elsewhere (McCormick et al.,1995). Plasma glucose levels were measured using Freestyle blood glucose meter (Therasense, Uppsala, Sweden). Liver glycogen was measured by the levels of glucose in hydrolyzed liver. Liver tissues were hydrolyzed with 2 M HCL for 4 hours. After 4 hours, samples were neutralized with 2 M NaOH and 1 M Tris (ph 7.4). Glucose levels from the hydrolyzed liver were normalized with DNA concentration contained in liver lysate.

***Ex vivo* tissue explant culture.** Neonatal (5- to 7-day-old) PER1::LUC transgenic mice were sacrificed and the brains were quickly removed. Following removal, brains were cooled and moistened with Gey's Balanced salt solution supplemented with 0.01 M HEPES and 36mM D-glucose. The brains were coronally sectioned in 400 μm thickness with a vibratome. The slices were then maintained on a culture insert membrane (Millicell-CM, Millipore, Bedford, MA, USA) and dipped into culture medium (50% minimum essential medium, 25% Gey's balanced salt solution, 25% horse serum, 36 mM glucose, and 100 units/ml aerosolized antibiotics) at 37 ℃.

The SCN slices were cultivated for two weeks before used in the experiments. For mature SCN slice culture, SCN from 5 week-old PER1::LUC transgenic mice were used. Instead of Gey's Balanced salt solution, Hank's balanced salt solution supplemented with 0.001 M HEPES and 1 mg/ml penicillin-streptomycin was used (Guilding et al., 2009). The SCN slices were cultivated for less than one week before used in the experiments.

Quantitative real-time bioluminescence monitoring on the SCN. The bioluminescence from the SCN slice cultures was monitored as reported previously with modification (Asai et al., 2001). The SCN slice culture of PER1::LUC mice was maintained in a sealed 35 mm petri dish with 1 ml of the culture medium (50% minimum essential medium, 25% Gey's balanced salt solution, 25% horse serum, 36 mM D-glucose, and 100 units/ml aerosolized antibiotics) containing 0.3 mM D-luciferin (Promega, Madison, WI, USA) at 36℃. The light emission was measured and integrated for 1 min at 10 min intervals, with a dish-type wheeled luminometer (AB-2550 Kronos-Dio; ATTO, Tokyo, Japan).

Statistical analysis. Period and phase measurements were calculated as previous reports (Abe et al., 2002; Yamazaki et al., 2002). Briefly, the original data (2-min bins) were smoothed by an adjacent-averaging method

with 1.67 hr running means. Bioluminescence data were detrended by subtracting a 24 hour running average from the raw data. The peak was calculated as the highest point of the smoothed data by using the CLUSTER8 (Veldhuis: University of Virginia, Charlottesville, VA), a statistical analysis program identifies significant interval value of peak and nadir. Peaks and amplitudes were normalized by dividing the difference of a peak and nearby nadir by average intensity of the basal section. The results from mRNA expression and metabolite levels were statistically evaluated by two-way ANOVA with Student's *t* test.

Table 1

Primer sequences for real-time RT-PCR

Target gene	Primer sequence
Bmal1	up: 5'-GGC CAT CAG TTA AGG TGG AA-3'
	dn: 5'-GGT GGC CAG CTT TTC AAA TA-3'
Clock	up: 5'-TTG CTC CAC GGG AAT CCT T-3'
	dn: 5'-GGA GGG AAA GTG CTC TGT TGT AG-3'
Rev-erbα	up: 5'-AGG GCA CAA GCA ACA TTA CC-3'
	dn: 5'-CAC AGG CGT GCA CTC CAT AG-3'
Rev-erbβ	up: 5'-CTG AAG AGT GAC CGC ACA CTA-3'
	dn: 5'-TAG TCA TGC CAG GAG CAC TG-3'
Per1	up: 5'-GTG TCG TGA TTA AAT TAG TCA G-3'
	dn: 5'-ACC ACT CAT GTC TGG GCC-3'
Per2	up: 5'-ATG CTC GCC ATC CAC AAG A-3'
	dn: 5'-GCG GAA TCG AAT GGG AGA AT-3'
Cry1	up: 5'-CTG GCG TGG AAG TCA TCG T-3'
	dn: 5'-CTG TCC GCC ATT GAG TTC TAT G-3'
Cry2	up: 5'-TGT CCC TTC CTG TGT GGA AGA-3'
	dn: 5'-GCT CCC AGC TTG GCT TGA-3'

up: upstream
dn: downstream

Table 2

Primer sequences for real-time RT-PCR

Target gene	Primer sequence
GLUT2	up: 5'-GTA CTC TTC ACC AAC TGG-3'
	dn: 5'-AAT AAA GCT GAG GCC AGC AA-3'
PEPCK	up: 5'-CTG GCA CCT CAG TGA AGA CA-3'
	dn: 5'-TCG ATG CCT TCC CAG TAA AC-3'
Glycogen Synthase2	up: 5'-CCA GCT TGA CAA GTT CGA CA-3'
	dn: 5'-ATC AGG CTT CCT CTT CAG CA-3'
GAPDH	up: 5'-CAT CCA CTG GTG CTG CCA AGG CTG T-3'
	dn: 5'-ACA ACC TGG TCC TCA GTG TAG CCC A-3'

up: upstream

dn: downstream

RESULTS

Altered cyclic PER1::LUC expression in SCN from 1 week-old mice.

I hypothesized that maternal stress can affect the circadian rhythm. I aimed to investigate the intrinsic defects of circadian clockwork and the function of SCN in the absence of systemic cue. To test this idea, I performed *ex vivo* tissue explant culture with PER1::LUC transgenic mice that carry a luciferase reporter gene under the control of the *mPer1* promoter (Asai et al., 2001). In this experiment, I compared the period and amplitude of PER1::LUC bioluminescence in the SCN from 1 week-old control and maternally stressed mice. Whereas the control group showed a robust oscillation in PER1::LUC expression (Fig. 1A), there were two patterns in the stressed group. About 67% (14 out of 20) of the maternally stressed group showed reduced PER1::LUC rhythm and 33% (6 out of 20) exhibited completely arrhythmic pattern (Fig. 1B and 1C). Next, I calculated the period and amplitude without the arrhythmic stressed group. Interestingly, the amplitude of the intensities of bioluminescence signals was decreased in stressed mice comparing to control mice (Fig 2A and 2B; 42.13 ± 2.63% for CTL, n=19; 24.98 ± 3.17% for STR, n=14). Furthermore, maternal stress evoked shortened period of PER1::LUC expression in the

SCN (Fig. 2C; 24.51 ± 0.06 hour for CTL, n=19; 24.26 ± 0.08 hour for STR, n=14).

Cyclic PER1::LUC expression in SCN from 5 week-old mice.

I used 5 week-old PER1::LUC transgenic mice to determine the prolonged effect of maternal stress on the circadian rhythm and SCN function. In contrast to SCN from 1 week-old mice, there were no arrhythmic patterns in the expression of PER1::LUC in the SCN from 5 week-old maternally stressed mice (Fig 3A and 3B) comparing to the SCN from 1 week-old maternally stressed mice which showed both abnormal and arrhythmic patterns. In case of SCN from 5 week-old mice, % values of amplitude in PER1::LUC expression from maternally stressed mice were still lower than that of control mice (Fig 4A and 4B; 13.98 ± 0.88% for CTL, n=7; 10.05 ± 1.60% for STR, n=7). However, periods of PER1::LUC expression were not changed (Fig 4C; 24.26 ± 0.05 hour for CTL, n=7; 24.26 ± 0.08 hour for STR, n=7).

Expression of clock genes in the local clock

The next set of experiments aimed to test the *in vivo* mRNA profiles of clock genes composing the core loop and the auxiliary loop by real-time

PCR. Because liver is a major organ that regulates various metabolic gene expressions, I examined whether liver-specific peripheral clockworks are affected by maternal stress. As shown in Fig. 5, two-way ANOVA revealed that the cyclic expression of some clock gene transcripts was decreased in the liver from the maternally stressed mice, especially Rev-erbα ($F_{(3,24)}$=253.24, p<0.0001 for maternal stress; $F_{(1,24)}$=17.38, p<0.001 for circadian time point; $F_{(3,24)}$=15.02, p<0.0001 for interaction) and Per1 mRNA expressions ($F_{(3,24)}$=57.24, p<0.0001 for maternal stress; $F_{(1,24)}$=10.43, p<0.001 for circadian time point; $F_{(3,24)}$=4.69, p<0.05 for interaction) at the peak times of their expression were significantly reduced. In addition, mRNA expression of Cry1 ($F_{(3,24)}$=31.05, p<0.0001 for maternal stress; $F_{(1,24)}$=9.90, p<0.01 for circadian time point; $F_{(3,24)}$=0.47, p=0.7069 for interaction) and Cry2 ($F_{(3,24)}$=4.55, p<0.05 for maternal stress; $F_{(1,24)}$=5.45, p<0.05 for circadian time point; $F_{(3,24)}$=0.49, p=0.6914 for interaction) was also decreased by the effect of the maternal stress

Effect of maternal stress on carbohydrate metabolism.

In the next experiment, I aimed to test physiological changes especially glucose metabolism in maternally stressed mice. To test this idea, daily oscillation of metabolic gene expression involved in glucose metabolism was examined by real-time PCR. Expression of phosphoenolpyruvate

carboxykinase (PEPCK) involved in gluconeogenesis was suppressed by maternal stress (Fig 6A; $F_{(1,24)}$=6.802, p<0.05 for maternally stressed mice; $F_{(3,24)}$=48.66, p<0.0001 for circadian time point; $F_{(3,24)}$=0.6577, p=0.5861 for interaction). Circadian expression of glycogen synthase2 (GS2) was increased (Fig 6B; $F_{(1,24)}$=0.04449, p=0.8347 for maternally stressed mice; $F_{(3,24)}$=28.31, p<0.0001 for circadian time point; $F_{(3,24)}$=4.464, p<0.05 for interaction) and glucose transporter2 (GLUT2) was also changed (Fig 6C; $F_{(1,24)}$=0.9306, p=0.3443 for maternally stressed mice; $F_{(3,24)}$=8.025, p<0.001 for circadian time point; $F_{(3,24)}$=9.793, p<0.001 for interaction) in the maternally stressed mice.

To examine whether this altered expression of metabolic genes that regulate glucose homeostasis accompany abnormal metabolites levels, I measured the daily rhythm of metabolic parameters related to glucose such as liver glycogen and plasma glucose. Although liver glycogen contents were slightly decreased in the maternally stressed mice (Fig 7A; $F_{(1,49)}$=10.32, p<0.001 for maternal stress; $F_{(3,49)}$=58.48, p<0.0001 for circadian time point; $F_{(3,49)}$=2.794, p=0.0500 for interaction), plasma glucose levels were not affected by maternal stress. Plasma glucose levels were fairly stable over the 24 hours (Fig 7B; $F_{(1,46)}$=0.1086, p=0.7432 for maternal stress; $F_{(3,46)}$=2.090, p=0.1145 for circadian time point; $F_{(3,46)}$=0.9733, p=0.4135 for interaction) when mice were fed *ad libitum*.

Figure 1. Two pattern of PER1::LUC oscillation in the maternally stressed mice. 30 minutes before nadir point is fixed as a starting point in each experiment. Representative record of bioluminescence of PER1::LUC oscillation in the control group (A) and Two patterns of PER1:LUC in the maternally stressed group are shown here. SCN from maternally stressed mice showed abnormal (B) and arrhythmic pattern (C) in the expression of PER1:LUC.

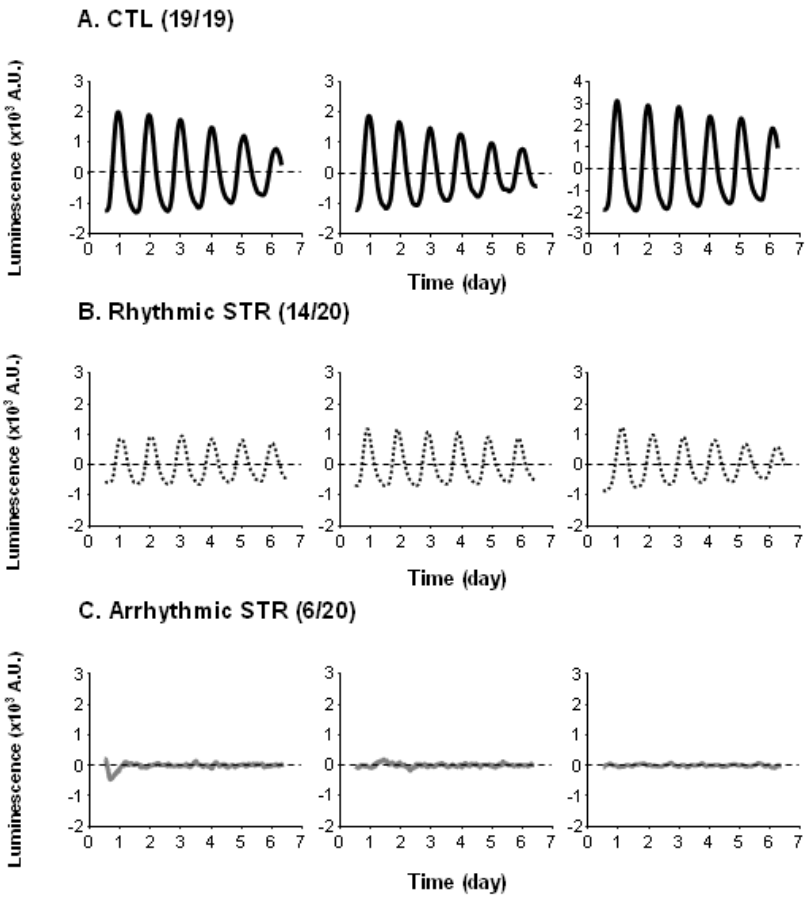

A. CTL (19/19)
Luminescence (x10³ A.U.)
Time (day)
B. Rhythmic STR (14/20)
Luminescence (x10³ A.U.)
Time (day)
C. Arrhythmic STR (6/20)
Luminescence (x10³ A.U.)
Time (day)

Figure 2. Effect of maternal stress on the circadian features of cycling PER1::LUC activities in the SCN. (A) A heat-map presentation voxels measured SCN from control and stressed mice. Red corresponds to the peak of bioluminescence and green to the trough. (B) Average periods in control and stressed mice. Peak-to-peak intervals during incubating were averaged, and the mean period ± SEM (hour) from independent sets of experiments were calculated. (C) Effects of maternal stress on the amplitude of PER1::LUC expression in the SCN from CTL and STR. (n=14 for CTL and n=19 for STR; *, $P<0.05$ and **, $P<0.01$ between CTL and STR).

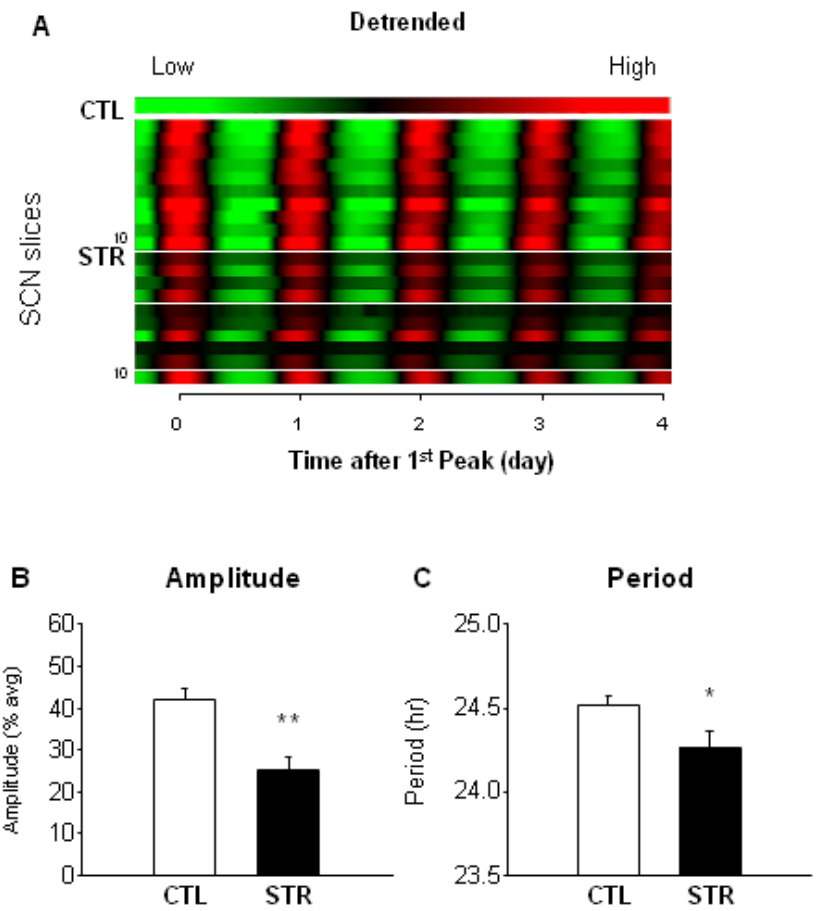

A
Detrended
Low
High
CTL
STR
SCN slices
0
1
2
3
4
Time after 1st Peak (day)
B
Amplitude
Amplitude (% avg)
60
50
40
30
20
10
0
**
CTL
STR
C
Period
Period (hr)
25.0
24.5
24.0
23.5
*
CTL
STR

Figure 3. PER1::LUC expression in the SCN from 5 week-old control and maternally stressed mice. 30 minutes before nadir point is fixed as a starting point in each experiments. A representative circadian profile of PER1::LUC expression in the mature SCN explant culture from CTL (A) and STR (B).

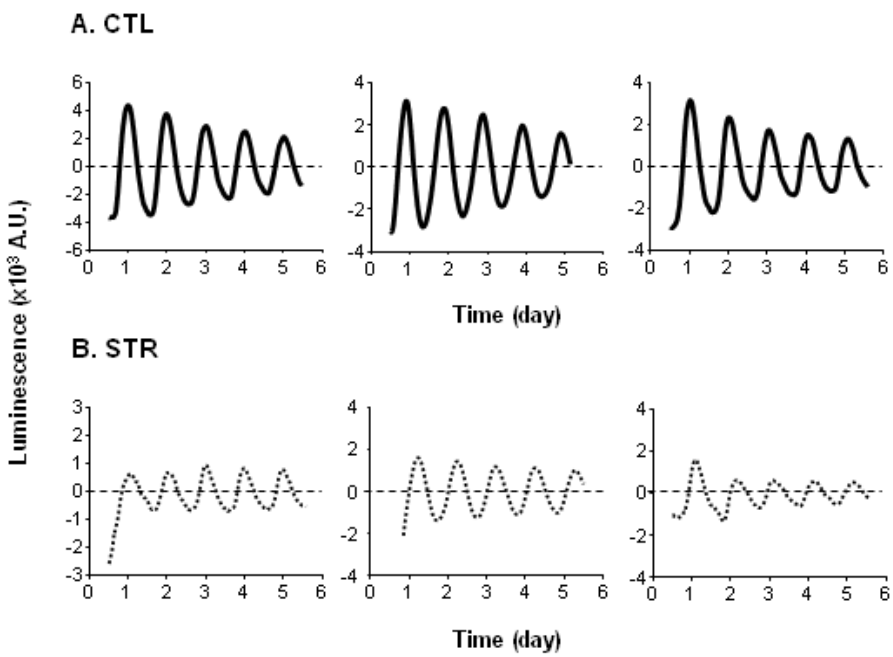

A. CTL
B. STR
Luminescence (x10³ A.U.)
Time (day)
Time (day)

Figure 4. Changes in circadian PER1::LUC expression in the SCN from 5 week-old maternally stressed mice. Bar charts summarizing effects of maternal stress on the rhythmic gene expression of PER1:LUC. (A) A heat-map presentation voxels measured SCN from control and stressed mice. Red corresponds to the peak of bioluminescence and green to the trough. Mean period (B) and amplitude (C) are expressed as mean ± SEM. (n=7 for CTL and STR; *, P<0.05 between CTL and STR).

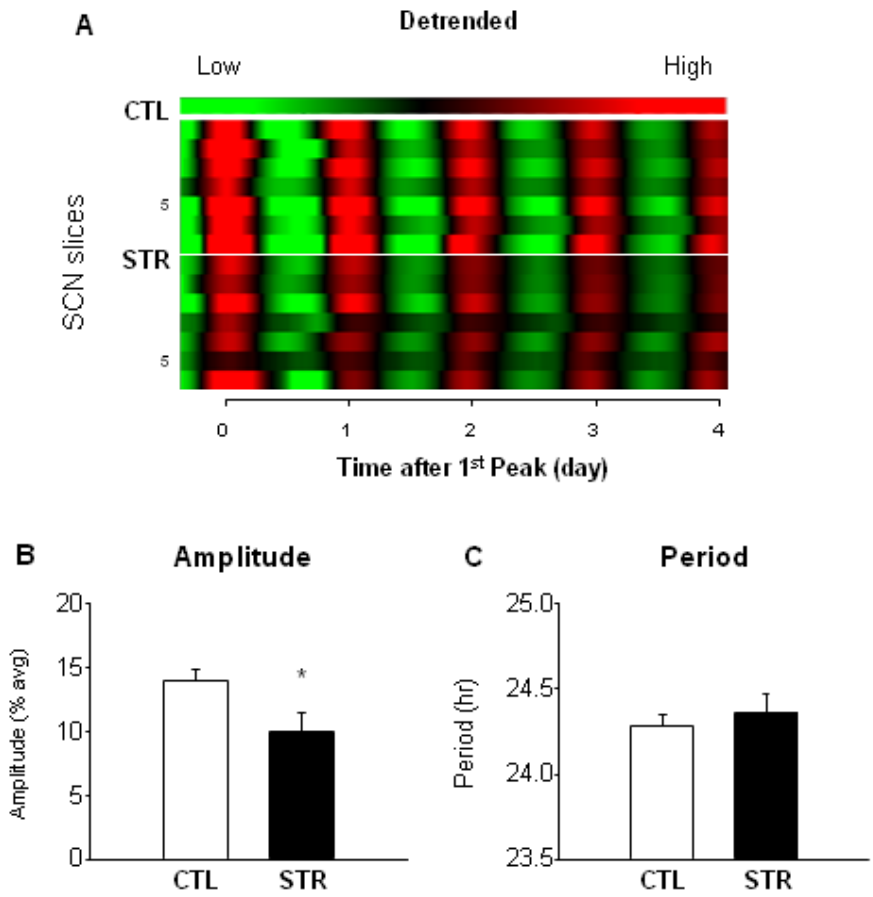

A
Detrended
Low
High
CTL
STR
SCN slices
5
5
0
1
2
3
4
Time after 1st Peak (day)
B
Amplitude
Amplitude (% avg)
20
15
10
5
0
*
CTL
STR
C
Period
Period (hr)
25.0
24.5
24.0
23.5
CTL
STR

Figure 5. Altered clock gene expression in liver local clock from maternally stressed mice. CTL and STR mice housed under DD conditions for 2 days were sacrificed at the circadian time 06, 12, 18 and 24. Clock gene mRNA expression profiles were obtained by real-time RT-PCR in Liver. Data were normalized with GAPDH and expressed as means ± SEM of A.U., where the mean CTL value at CT24 is defined as 1 (n=4, *, $P<0.05$ and **, $P<0.01$ vs. CTL at the same time points).

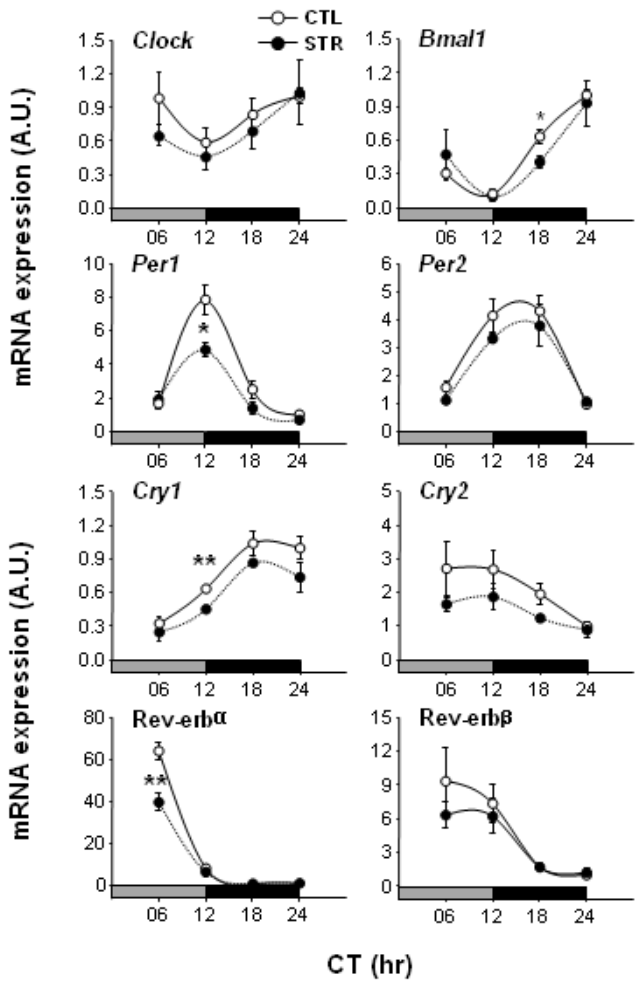

CTL
STR
Clock
Bmal1
Per1
Per2
Cry1
Cry2
Rev-erbα
Rev-erbβ
mRNA expression (A.U.)
mRNA expression (A.U.)
CT (hr)
06
12
18
24

Figure 6. Circadian expression of metabolic genes in the liver. Metabolic gene mRNA expression profiles of PEPCK (A), GS2 (B) and GLUT2 (C) were determined by real-time RT-PCR in Liver. All mRNA levels were normalized with GAPDH and expressed as means ±SEM of A.I., where the mean CTL value at CT24 is defined as 1 (n=4, *, $P<0.05$ and **, $P<0.01$ vs. CTL at the same time points).

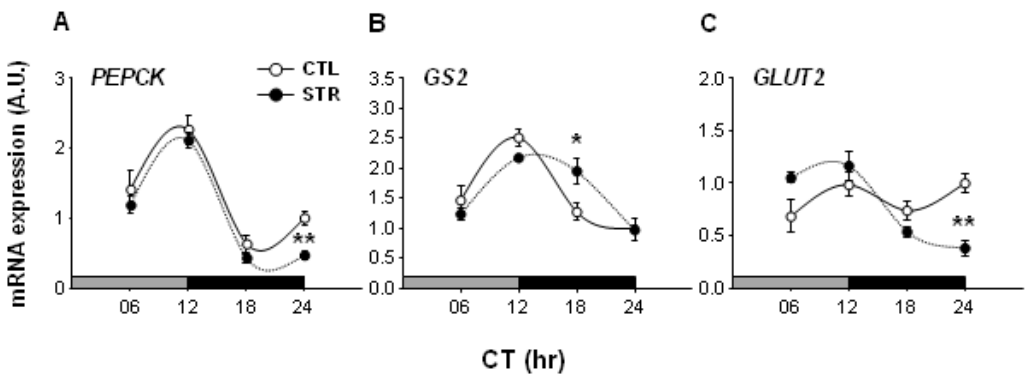
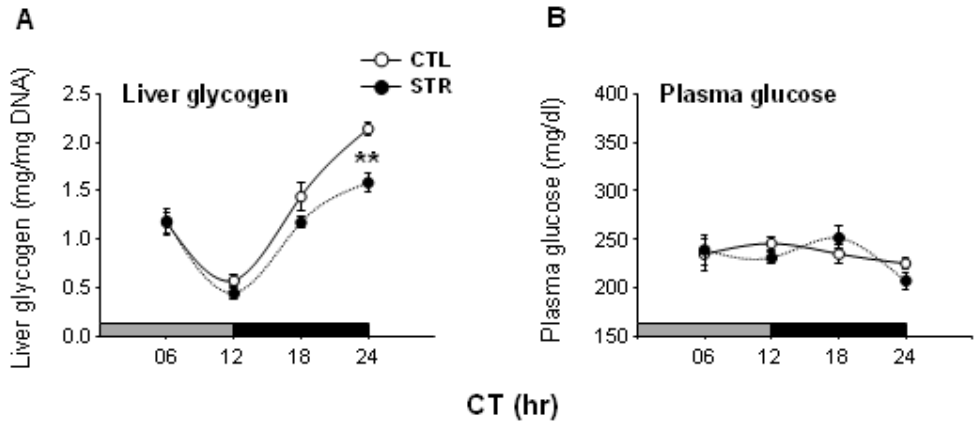

A
B
C
PEPCK
GS2
GLUT2
CTL
STR
mRNA expression (A.U.)
CT (hr)
06
12
18
24
*
**

Figure 7. Circadian rhythm of metabolic parameters. CTL and STR mice were entrained to a 12:12 LD photoperiodic cycle. After 2days of constant darkness (DD) condition, mice were sacrificed at the indicated time. Daily rhythm of liver glycogen (A) and plasma glucose (B) were measured. All data were expressed as mean ± SEM (n=6~8 for CTL and n=4~7 for STR; **, P<0.01 between CTL and STR).


A
B
CTL
STR
Liver glycogen
Plasma glucose
Liver glycogen (mg/mg DNA)
Plasma glucose (mg/dl)
2.5
2.0
1.5
1.0
0.5
0.0
400
350
300
250
200
150
**
06
12
18
24
CT (hr)

DISCUSSION

The present study demonstrated that maternal stress affected circadian rhythm at the early postnatal days as depicted by altered period and amplitude in PER1::LUC expression and these effects were recovered later when animals become mature and reached to adult stage. Furthermore, expression of liver-specific molecular clock and metabolic genes involved in glucose metabolism were altered but it could not cause significant changes in glucose metabolism at adult stage.

In the rat, maternal stress caused advanced timing of the increase in corticosterone secretion and locomotor activity (Koehl et al., 1997; Maccari et al., 1997). Therefore, it is worthwhile to examine the expression of molecular clock genes involved in circadian rhythm. In the *ex vivo* SCN tissue explant culture, the SCN from 1 week-old control mice showed a robust rhythm in PER1::LUC expression as shown in Fig. 1A. In contrast, in maternally stressed mice there were two patterns in PER1::LUC expression: one of them exhibited reduced PER1::LUC expression levels and the other showed arrhythmic patterns. Because there were no arrhythmic patterns in the control group, it was not a technical problem in the explant tissue culture. In addition, maternally stressed mice exhibited shortened period of PER1::LUC expression and lower amplitude of the

bioluminescence signals comparing to the control group in SCN from 1 week-old mice. *Ex vivo* experiment revealed that maternal stress causes significant defect of circadian rhythm in 1 week-old mice. According to the previous study, serving a functional neural network as a circadian rhythm generator is formed at the first 10 days after birth, and this is when the SCN neurons undergo a rapid development (Moore and Bernstein, 1989). Therefore, completely arrhythmic patterns of SCN neurons in maternally stressed mice may be the result of developmental retardation. Thus, at postnatal day 7, the SCN in the arrhythmic stressed group may function immaturely or some SCN may be entirely nonfunctional. However, it should be noted that there were no arrhythmic patterns in PER1::LUC expression of SCN from 5 week-old maternally stressed mice (Fig. 3). In these mice, although the amplitude was still reduced, the period of PER1::LUC expression seemed normal in the SCN derived from maternally stressed mice. Based on such different patterns of the SCN from 1 week-old and 5 week-old mice, it can be postulated that maternal stress affects the SCN function and thereby some maternally stressed offspring showed arrhythmicity as well as abnormal circadian rhythm in their early life and these effects may recover later.

Along with the central clock, liver peripheral clockwork was also affected by maternal stress. Per1 and Rev-erbα showed significantly reduced amplitude of circadian mRNA levels. However, the peak time did

not change, that means the period did not change, in accordance with *ex vivo* experiment in 5 week-age mice. The master circadian pacemaker residing in the SCN can reinstall and affect the liver oscillator probably by synchronizing the circadian molecular clockwork (Guo et al., 2005). Thus, these changes could be the consequence of the malfunction in SCN or maternal stress could have caused altered expression of common clock components in both SCN and liver.

Abnormal metabolism in the liver-specific molecular clock gene mutants indicates that liver clock has a physiologically important function in metabolism. For example, clock genes in the liver contribute to glucose homeostasis by driving a daily rhythm of hepatic glucose export (Lamia et al., 2008). Because previous studies on the metabolic dysregulation in maternally stressed mice were performed only at a single time point (Lesage et al., 2004; Mairesse et al., 2007), the present study examined temporal changes in daily oscillation of metabolite levels and metabolic gene expressions. As shown in Fig. 6, mRNA expression profiles of metabolic genes involved in glucose metabolism including PEPCK, GS2 and GLUT2 were also changed, implying that altered liver clock by maternal stress causes altered regulation in metabolic gene expression. Abnormal metabolic gene expression generally accompanies metabolic dysreugulation. In agreement with the previous findings, daily rhythm of glycogen levels was slightly reduced in maternally stressed mice (Cleasby

et al., 2003) around the same time points when the metabolic gene expressions were disrupted. However, plasma glucose levels were not changed. When nocturnal mice were fed *ad libitum*, blood glucose remained fairly stable throughout a circadian cycle (Yoon et al., 2012). Also blood glucose are controlled by various factors such as corticosterone, leptin and insulin (Andrews and Walker,1999; Saltiel and Kahn, 2001). Thus, basal levels of plasma glucose were not significantly changed at the adult stage under normal feeding even though received prenatal stress during pregnancy.

In conclusion, the present study demonstrated that maternal stress causes abnormal rhythmicity of SCN in the early age, which resulted in altered expression of liver clock genes and metabolic genes related glucose metabolism. Despite of these changes, glucose homeostasis was not significantly altered at the adult stage. These results suggest that maternally stressed mice probably adjust well to normal feeding and their environment also.

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국문초록

임신한 어미의 주변환경은 새끼에게 장기적인 영향을 줄 수 있으며, 임신한 어미를 통한 스트레스가 해마 (hippocampus)나 편도체 (amygdala)와 같은 중요한 뇌 영역에 미치는 영향 결함을 가져온다는 사실은 잘 알려진 사실이다. 그러나, 모체를 통한 스트레스가 일주기 리듬 (circadian rhythm) 조절에 핵심적인 역할을 하는 시신경교차상핵 (suprachiasmatic nucleus, SCN) 에 미치는 영향에 대한 연구는 아직 미진한 상태이다. 따라서 본 연구는 모체를 통한 스트레스가 일주기 리듬에 미칠 수 있는 영향과 함께 이들의 새끼에게서 나타날 수 있는 생리학적인 연관성을 알아보고자 하였다. 1 주령 PER1::LUC 형질전환 쥐의 SCN 조직에서는 PER1::LUC의 리듬이 1 주일 이상 유지되지만, 모체를 통한 스트레스를 받고 태어난 쥐에서는 주기가 짧아져있고, 진폭이 감소해 있으며 리듬이 완전히 사라진 경우도 관찰할 수 있었다. 그러나, 5 주령의 SCN 조직에서 진폭은 여전히 감소해 있는 반면, 짧아진 주기와 리듬이 사라진 양상은 회복되어있는 것을 볼 수 있었다. 또한, 태내 스트레스를 받고 태어난 성체의 간에서의 분자생체시계 유전자의 발현 변화와 더불어 포도당 대사를 조절하는 유전자들의 일주기적 발현양상에도 변화가 보였다. 하지만, 간에서의 글리코겐의 감소에도 불구하고 혈중포도당 농도에는 변가 없었는데, 정상적인 먹이조건에서는 태내 스트레스에 의한 영향으로 포도당의 항상성이 망가지지 않는다고 할 수 있다. 결론적으로 태내 스트레스를 받고 태어난 쥐는 자라면서 태내 스트레스에 의한 일주기 리듬 변조를 극복하기 위한 방향으로 주변환경에 적응했다고 볼 수 있다.

주요어: 모체를 통한 스트레스 (maternal stress), 일주기 리듬 (circadian rhythm), 시신경교차핵 (suprachiasmatic nucleus, SCN), 물질대사