It is well established that the mammalian circadian system consists of pacemaker cells in the suprachiasmatic nuclei (SCN). The mouse has become increasingly important in understanding the circadian timing system, due to the availability of mutant animals with abnormal circadian rhythms. In the present paper, we describe the organization of the mouse SCN, comparing the wild type and Clock mutant animal, with a special focus on those peptides bearing an upstream E-box element (vasopressin, vasoactive intestinal peptide, cholecystokinin, and substance P). To this end, we describe the distribution of the foregoing SCN peptidergic cell types as well as gastrin-related peptide, calretinin, calbindin, somatostatin, neurotensin and retinal input to the SCN (determined by both tract tracing and fos-immunoreactivity in response to a light pulse). The Clock mutant mouse has decreased expression of vasopressin mRNA and protein in the SCN, with normal patterns of expression elsewhere in the brain. No other differences were detected between the Clock mutant and the wild type mouse. The results are consistent with the hypothesis that there are multiple regulatory elements of clock-controlled genes in the SCN.

Key words: Cholecystokinin; Circadian rhythms; Clock mouse; Suprachiasmatic nucleus; Vasoactive intestinal polypeptide; Vasopressin

Introduction

All organisms display daily variations in physiological and behavioral responses, most of which continue in the absence of external environmental cues. The circadian system responsible for producing these 24 h rhythms measures time and synchronizes the organism’s internal processes with the daily events in its environment. In mammals, circadian rhythms are known to be controlled by a clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus. Numerous reports have established that SCN-lesioned animals become arrhythmic, and that when the SCN is transplanted from one individual to another, the host animal expresses circadian rhythmicity with the period of the donor (reviewed in [1]).

In rodents, each SCN contains 8–10,000 cells, comprising several subpopulations that differ in cell size, afferent input, efferent output, transmitter and peptide content (reviewed in [1]). Because of the availability of genetic information, the mouse has become an important subject of circadian studies. Therefore, the first goal of the present experiments was to map the distribution of peptidergic cells, including vasopressin (VP), vasoactive intestinal peptide (VIP), gastrin-related peptide (GRP), cholecystokinin (CCK), calretinin, calbindin, somatostatin (SS), substance P (SP), neurotensin (NT), neuropeptide Y (NPY) and retinal input to the SCN (using tract tracing and c-fos immunoreactivity in response to a light pulse).

The Clock mutation in the mouse confers a behavioral phenotype that differs from that of wild type mice [2]. Heterozygous Clock mutants express a free-running activity period of about 24.4 h, while the period of the parent strain is about 23.3 h. Clock homozygotes have a free-running period of about 28 h in the first days after housing in constant darkness (DD), and then they become arrhythmic. The clock gene encodes a transcription factor that contains a protein-protein interaction sequence...
(PAS domain) as well as a DNA binding sequence (basic helix–loop–helix domain) [3]. Through the PAS domain, the CLOCK protein forms a heterodimer with another transcription factor expressed in the SCN, BMAL-1 [4]. The CLOCK–BMAL-1 heterodimer binds to a consensus DNA sequence, known as the E box, found in the 5′ flanking region of circadian genes, such as mPerl [4]. Binding of the CLOCK–BMAL-1 heterodimer to the E-box activates transcription of target genes [4]. The Clock mutation results in an altered protein that forms a heterodimer with BMAL-1, but is unable to activate transcription [4]. Expression of the mutant CLOCK protein is therefore thought to interrupt the transcription–translation feedback loop underlying circadian pacemaker function [5]. Consistent with this effect of the mutant CLOCK protein, mPerl mRNA levels are diminished in the SCN of Clock mutant mice [6].

The second objective of the present study was to assess differences between wild type and Clock mutant mice. Of special interest in this context were the four peptides that have upstream E-box regulatory elements (VP, VIP, CCK and SP; see Discussion for further details).

Materials and Methods

Subjects and housing: Adult mice were bred from a colony of BALB/c X C57BL/6J clock+/− (gift of M. Vitaterna and J. Takahashi). Experimental animals expressed a free-running period of locomotor activity shorter than 23.5 h (wild-type mouse) or became arrhythmic after 2–3 weeks in DD (homozygous Clock mutants). The room was kept at about 23°C. Animals were housed in translucent propylene cages (45 × 24 × 15 cm) and provided with ad lib access to food and water. The animals were born and raised in a 14:10 h light:dark cycle. At about age 2 months, they were transferred to constant darkness (DD) for 2–3 weeks and placed in cages (35 × 21 × 18 cm) equipped with a running wheel (diameter 12.7 cm). Locomotor activity was monitored continuously using a computer-based data acquisition system (Dataquest, Data Sciences, St Paul, MN). A dim red light, which generates <1 lux (Delta 1, Dallas, TX) allowed for animal maintenance. Once their phenotype was determined, mice became arrhythmic after 2–3 weeks in DD (homozygous Clock mutants). Experimental animals were anesthetized (pentobarbital: 200 mg/kg) and perfused intracardially with 50 ml 0.9% saline followed by 100 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Brains were post-fixed for 18–24 h at 4°C, cryoprotected in 20% sucrose in 0.1 M phosphate buffer overnight. Alternate 40 μm sections were processed free-floating with two sections per well, one wild type and one Clock mutant. A cut was made in the cortex of wild type mice in order to distinguish the animals. The following primary antibodies and concentrations were used: rabbit polyclonal VP (1:10 000), VIP (1:10 000), GRP (1:3000), CCK (1:5000), SS (1:2000), SP (1:10 000), NT (1:2000), NPY (1:10 000) all from Incstar, calretinin (1:5000; Chemicon), c-fos (1:10 000; Calbiochem), goat polyclonal choleragenoid (1:15 000; List Biological, Campbell, CA), mouse monoclonal calbindin (1:20 000; Sigma), with a modified avidin–biotin–immunoperoxidase technique. The chromogen used was the SG substrate (Vector). The sections were coverslipped with permount.

Perfusion and immunocytochemistry: Mice were anesthetized (pentobarbital: 200 mg/kg) and perfused intracardially with 50 ml 0.9% saline followed by 100 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Brains were post-fixed for 18–24 h at 4°C, cryoprotected in 20% sucrose in 0.1 M phosphate buffer overnight. Alternate 40 μm sections were processed free-floating with two sections per well, one wild type and one Clock mutant. A cut was made in the cortex of wild type mice in order to distinguish the animals. The following primary antibodies and concentrations were used: rabbit polyclonal VP (1:10 000), VIP (1:10 000), GRP (1:3000), CCK (1:5000), SS (1:2000), SP (1:10 000), NT (1:2000), NPY (1:10 000) all from Incstar, calretinin (1:5000; Chemicon), c-fos (1:10 000; Calbiochem), goat polyclonal choleragenoid (1:15 000; List Biological, Campbell, CA), mouse monoclonal calbindin (1:20 000; Sigma), with a modified avidin–biotin–immunoperoxidase technique. The chromogen used was the SG substrate (Vector). The sections were coverslipped with permount.

Immunoistochemical data analysis: Brain sections were captured using a CCD video camera (Sony XC77) attached to a light microscope (Olympus BH-2) using NIH Image (1.61). Such images were used for drawing the schematics in Fig. 1. Sections were examined under the light microscope and VP,
FIG. 1. Schematic representing the peptidergic organization of the wild type BALB/c × C57BL/6J mouse SCN at its rostral (row 1), second and third quadrants (rows 2 and 3) and caudal (row 4) aspects. The Clock mutant mouse has a similar peptidergic organization except for VP. Dashed lines indicate boundaries of the nucleus based on Nissl-stained sections.
CCK and VIP cells were counted by two independent investigators, blind to the experimental conditions (identifiers on the slides were covered). The number of VP, VIP and CCK profiles was counted on alternate sections throughout the SCN. Cell number was determined using the Abercrombie [8] correction factor calculated as follows: \[ N = \frac{2nT}{T + D} \], where \( N \) is the corrected cell number, \( n \) is the uncorrected cell number multiplied by 2 (alternate sections were used), \( T \) is the section thickness, and \( D \) is the mean cell diameter calculated from the perimeter of the SCN cells. To estimate cell diameter, the perimeter of the VP, VIP and CCK cells in SCN was measured using the Image program (NIH, v1.61). For VP and VIP, 40 cells from four wild type and four Clock mutant mice were measured. For CCK, 20 cells from two wild type and two Clock mutant mice were measured; there were no differences between wild-type and mutant groups, and the data were combined (mean (±s.e.m.) VP cell diameter 7.24 ± 0.10 μm: wild type 7.16 ± 0.15, Clock mutant 7.33 ± 0.13; mean VIP cell diameter 8.20 ± 0.17 μm: wild type 8.40 ± 0.16, Clock mutant 8.13 ± 0.69; mean CCK cell diameter 9.71 ± 0.14 μm: wild type 9.64 ± 0.22, Clock mutant 9.78 ± 0.19). We also sampled the number of VP cells in the PVN and SON. For the PVN, the VP cells were counted on four alternate sections in the medial PVN. For the SON, the number of VP cells was counted on five alternate sections in the rostral SON. All the data were analyzed by ANOVA, with Fisher’s PLSD for post hoc comparisons between groups.

In situ hybridization method: Mice were anesthetized with carbon dioxide and sacrificed by decapitation. Brains were removed and frozen on dry ice and stored at −80°C until used. Coronal sections were cut at 14 μm on a cryostat and mounted on Superfrost Plus (Fisher) slides. The sections were then fixed in 4% paraformaldehyde, followed by two rinses in 0.1 M phosphate buffer and subsequent treatment with 0.25% acetic anhydride. Sections were then dehydrated through graded alcohols followed by delipidation in chloroform. A 63 bp oligonucleotide probe, complementary to exon 2 (bp 2814–2877) of the mouse vasopressin gene, was end-labeled with [35S]dATP using Terminal Transferase (Boehringer Mannheim). Unincorporated radiolabeled nucleotides were removed by Quick Spin Columns (Boehringer Mannheim). Probe was diluted in standard hybridization buffer containing 62.5% formamide to 100,000 c.p.m./slide. Probe (~100 ml) was applied and slides coverslipped with Parafilm, sealed and incubated overnight at 37°C in a humid chamber. On the following day coverslips were removed and the slides washed once in 1× standard sodium citrate (SSC) buffer at room temperature, twice in 1× SSC at 50°C, and finally twice (1 h each) in 1× SSC at room temperature. Slides were air dried then dipped in warmed NBT2 emulsion (VWR) and exposed at 4°C for either 5 days or 2 weeks.

Prior to capturing images, and to facilitate silver grain counting on nissl-stained (cresyl violet) sections, a Wratten 47B filter was placed between the light source and objective once the SCN was visualized. Images were captured using a Nikon Microphot FX microscope fitted with a Dage MTI CCD camera and Scion LG-3 frame grabber in a Macintosh G3 computer.

In situ hybridization data analysis: Three sections, including rostral, mid and caudal regions of the SCN, were analyzed per animal. For the SCN, the total number of silver grains over an area encompassing the entire portion of one-half of the SCN was first converted to pixels and the subsequent total pixel number was determined using the NIH Image program. Non-specific signal (i.e. background) was subtracted from a region not containing VP message (same section) and of identical size to that used for SCN analysis. For PVN/SON analysis, the area of silver grains (pixel area) over individual cells was calculated in cells randomly sampled from three different tissue sections. Background correction was performed for each cell on a tissue region not expressing VP mRNA. Two-way ANOVA was used to test first for rostral-caudal differences in SCN VP mRNA and then for genotype, time of day and interactive effects.

Results

Peptidergic organization: The peptidergic organization of the wild type mouse SCN is shown in Fig. 1. VP-ir cells and fibers form a sphere around the center of the SCN, with many cells in the rostral and caudal region. The VIP-ir cells are located in the ventral SCN, with VIP-ir fibers densely distributed throughout the nucleus and extending dorsally along the periventricular region. The GRP-ir cells are distributed similarly to the VIP-ir cells but are much less numerous, while GRP fibers are localized to the borders of the SCN. The distribution of CCK-ir cells and fibers is similar to that of VP-ir cells and fibers. Calbindin-ir cells are found in the ventral SCN, with a distinct cluster of cells in the dorsolateral region. Calbindin-ir cells are dispersed throughout the SCN. SS-ir cells and fibers form a sphere just around the SCN boundaries, with fibers extending dorsally along the periventricular region. SP-ir fibers surround the SCN; no SP-ir cells were
detected. NT fibers also surround the SCN and are densest in the medial SCN near the third ventricle; the fibers extend dorsally in the periventricular region where they are denser medially and laterally. NPY fibers are most dense in the ventral and central parts of the SCN, and sparser in the dorsal and lateral SCN. Retinal fibers course throughout the SCN and extend dorsally along the periventricular region.

Light-induced c-fos-ir occurs in the ventral, lateral and dorsolateral SCN, avoiding the medial/mediodorsal SCN.

Comparison wild type-Clock mutant: With the exception of VP, there were no detectable differences in distribution or number of labeled cells between the wild type and the Clock mutant mouse (this conclusion is constrained by the limited number of animals examined for those peptides where we saw no differences in the preliminary analyses). The number of VP-positive cells in the SCN were decreased in mutant mice (Fig. 2, Fig. 3; genotype × circadian time ANOVA: genotype F = 15.92 (1,18); p < 0.001; circadian time F = 5.8 (1,18) Fisher PLSD; p = 0.02; genotype × circadian time F = 7.6 (1,18); p = 0.13), but not in the PVN (Genotype × circadian time ANOVA: genotype F = 0.42 (1,16); p = 0.53; circadian time F = 0.01 (1,16); p = 0.91; genotype × circadian time F = 3.6 (1,16); p = 0.08). The difference between wild type and mutant animals was most dramatic at ZT04, when VP expression is normally elevated. Restated, wild type animals have a daily rhythm of VP expression, while there is no such rhythm in the mutant animals (Clock mutant ZT04 vs ZT20, p = 0.80 Fisher PLSD). The decreased VP expression in SCN does not appear to be a reflection of a general decline in peptide synthesis as there were no obvious differences between WT and mutant animals in the other peptides we examined. Furthermore, there were no detectable differences in the number of VIP-positive cells (Fig. 2, Fig. 3; genotype × circadian time ANOVA; genotype F = 0.08 (1,16); p = 0.78; circadian time F = 0.88 (1,16); genotype × circadian time F = 1.6 (1,16); p = 0.23), or in the number of CCK cells (wild type 55.5 ± 17.5; Clock mutant 68.8 ± 30.3; genotype × circadian time ANOVA: genotype F = 0.19 (1,4); p = 0.69; circadian time F = 1.13 (1,4); p = 0.35; genotype × circadian time F = 2.69 (1,4); p = 0.18.

mRNA: The difference between groups in number of immunopositive VP cells was also reflected in VP mRNA levels. Thus, VP mRNA was more than 2-
fold higher in the SCN of wild type than in Clock mutants at ZT09, the time of peak VP mRNA expression (Fig. 4, Fig. 5). While there was a clear day-night difference in wild type animals, no significant diurnal difference was detected in VP mRNA in Clock mutant mice (genotype × circadian time ANOVA: genotype F = 69.16 (1,11); p = 0.0001; circadian time F = 41.36 (1,11); p = 0.0002; genotype × circadian time F = 17.05 (1,11); p = 0.003; Clock mutant ZT04 vs ZT20, p > 0.05 Fisher PLSD). Furthermore, the difference between wild type and clock mutant mouse was seen throughout the SCN, and was not restricted to a particular rostral-caudal level (region × genotype ANOVA: F = 0.86 (1,18); p = 0.37). VP mRNA levels in the SON and PVN did not differ between either wild type or Clock mutant animals, and no day-night difference was observed (genotype × circadian time ANOVA for SON: genotype F = 0.004 (1,11); p = 0.95; circadian time F = 0.36 (1,11); p = 0.57; genotype × circadian time F = 3.49 (1,11); p = 0.1; genotype × circadian time ANOVA for PVN: genotype F = 0.05 (1,11); p = 0.88; circadian time F = 0.04 (1,11); p = 0.86; genotype × circadian time F = 0.09 (1,11); p = 0.93).

Discussion

Overview of the mouse SCN peptidergic organization and retinal input: While VP, VIP, CCK, NPY, and retinal input have a similar distribution in mouse, rat, and hamster species (see Results), in several other respects, the organization of the mouse SCN [9] is similar to that of the rat, and somewhat different from that of the hamster [1,10]. VP cells ring the SCN, with many cells in the rostral and caudal region. In rat and mouse, GRP cells are located in the ventral SCN. In hamsters, they lie in the core region of the SCN, in a subregion characterized by GRP, SP and calbindin cells [10]. The distribution of calretinin is similar in rats and mice, with a greater concentration of immunoreactive cells in the ventral and the dorsolateral SCN regions. In hamsters, calretinin cells are sparser, and are found throughout the SCN. The distribution of calbindin in the mouse also resembles that of the rat; immunostained cells are seen throughout the nuclei, and this is unlike the hamster where some cells are found in the rostral dorsolateral SCN and a large group of cells are densely packed in the core of the SCN. SP cells were not detected in the SCN, and SP fibers occurred outside the SCN. In the rat, SP cells are located in the central and ventral SCN [11] and fibers form a plexus in the ventral SCN in addition to surrounding this nucleus [12]. In the hamster, a group of SP cells exist in the core of the SCN, and fibers surround the SCN [10,11]. Somatostatin cells are present at the border of the mouse SCN. In the rat, they are more concentrated to the dorsomedial SCN [13]. Light-induced c-fos occurs in the ventral
and lateral SCN, and resembles the distribution in the rat, but is different in the hamsters where the densest label is in the core of the SCN [1,10]. However, unlike the rat where retinal input is limited to the ventral portion of the SCN, retinohypothalamic fibers are found throughout the mouse SCN in both ventral and dorsomedial portions.

Wild type vs Clock mutant mouse: The only obvious difference detected between the wild type and Clock mutant mouse is a decreased expression in the latter of VP protein and mRNA in the SCN (but see Shearman and Weaver [14] for evidence of differences in c-fos mRNA in response to a light pulse). No differences were seen in other VP-ergic hypothalamic cells. Consistent with a recent report [6], both the number of VP-immunopositive cells and the levels of VP mRNA were significantly decreased in the SCN of Clock mutant animals compared to control, wild-type mice. While daytime VP mRNA levels in the SCN of wild-type mice were more than twice that of night levels, homozygote Clock mutant mice displayed no significant day–night difference in SCN VP mRNA levels. The lack of day–night difference in mutant mice persisted even when exposure time of the autoradiograms was increased to amplify the sensitivity of in situ hybridization signal detection.

Reduced VP mRNA has also been reported in the Tau hamster, another circadian rhythm mutant, compared to wild type hamsters [15], although the magnitude of the difference is less than is seen in the Clock mutant (vs wild type) mouse. The molecular basis for the Tau mutant phenotype has not yet been identified. The difference in behavioral phenotype between the mutant mouse and hamster, with the former but not the latter becoming arrhythmic in DD, suggests that different mechanisms mediate the altered circadian responses. In the Tau mutant hamster, evidence as to whether there are alterations in VIP mRNA is controversial, as both higher [16] and lower [15] values have been reported.

Vasopressin, CCK and VIP are peptides characteristic of the SCN of all mammals studied to date.
The absence of any difference in CCK, VIP, or in other peptidergic expression between Clock mutant and wild type mice shows that a general decrease does not occur in neuropeptide expression in the SCN of Clock mutant mice. There is an upstream consensus E box regulatory sequences (5’-CANNTG-3’) on the VIP, VP, CCK and SP genes, and the latter three have the specific E box (CACGTG) shown to bind BMAL1 and CLOCK. Examination of the genomic sequence of mouse VP [17] reveals a CACGTG E-box in the 5’ region, approximately 185 bp upstream from the transcription start site (GenBank accession number M88354). Nuclear run-on assays indicate that the VP rhythm in the SCN is due to changes in transcriptional activity [18].

CCK has a CACGTG E-box situated 91 bp upstream of the transcription initiation site (GenBank accession number L29399). Although a rhythm in CCK protein concentration has been shown in the rat nucleus accumbens and striatum [19], a diurnal rhythm (in LD cycles) in CCK was not seen in rat SCN [20]. The rat SP gene is known to have a consensus E box (CAGATG), and also a CACGTG E box [21]. SP protein, unlike VP, does not cycle in the rat SCN in either LD or DD [22]. The VIP gene also has an upstream domain that contains several consensus E-boxes (GenBank accession numbers X74297, X02341, U58468, M36634), but no CACGTG E box. VIP mRNA expression, at least in the adult SCN, is not regulated in a circadian fashion. In constant darkness (DD), circadian rhythms in VIP are seen in young animals, and diurnal but not circadian rhythms persist in adults [23] (reviewed in [7]).

An E box element in the VP gene is necessary for CLOCK–BMAL1 heterodimers to mediate rhythmic transcription of VP [6]. In the Clock mutant mouse an altered CLOCK protein affects the transcription–translation feedback loop underlying circadian function. This accounts for the lack of rhythmic VP expression in the Clock mutant mouse. CCK and SP genes have the CACGTG E box, but no difference was seen between wild type and clock mutant mice in either CCK cells or SP immunoreactivity, suggesting the presence of other regulatory elements. The VIP gene has consensus E boxes, but not the specific E box shown to bind BMAL1 and CLOCK.

In Clock mutant mice, a decrease in VP mRNA and protein expression is seen in the SCN but not in other VP-ergic cells such as those of the PVN or SON (see also [6]). If the decrease is due to a direct effect of the mutated CLOCK protein, then it may be limited to cells that normally co-express VP and CLOCK. While CLOCK has been described as being present in cells throughout the rostral-caudal extent of the mouse SCN [4], there is as yet no evidence as to the peptidergic phenotype(s) of CLOCK-expressing cells in the SCN or elsewhere.
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However, preliminary in situ hybridization data regarding the distribution of Clock mRNA in the human hypothalamus shows its presence in both the PVN and SON, as well as the SCN [24] and based on their location, these are very likely to be VP-ergic.

If CLOCK and VP are indeed co-expressed in SON and PVN cells, as well as in the SCN, then the regional specificity we observed may be due to differences between these nuclei in other transcriptional regulators of VP. For example, VP expression in the SON or PVN may be due to site-specific transcription factors that compensate for the lack of activity of the mutated CLOCK protein. The presence of a constitutively active positive transcriptional regulatory factor in the PVN and SON might also explain why VP mRNA is not rhythmic here (see Fig. 4). There have been few studies to date examining possible cellular differences in transcriptional regulation of VP gene expression between the SCN and SON/PVN [25], but this would be a fertile area for future investigation.

Functions of VP: An important unanswered issue is the functional significance of VP. The presence of circadian rhythms, both in a light-dark cycle and in constant light, in VP-deficient Brattleboro rats has been taken as evidence that VP is not necessary for the expression of circadian rhythmicity [26]. Furthermore, SCN grafts derived from Brattleboro donors are able to restore rhythms to SCN-lesioned rats [27]. Nevertheless, a very large body of evidence, based on assay, removal and replacement studies, implicates VP in the modulation of circadian rhythms. First, there is a correlation between the VP secretion and the components of circadian rhythms. Several circadian responses are associated with numbers of VP-ir neurons in different mouse lines and in aged voles [28,29]. In addition, rhythmic N-acetyltransferase levels are dampened in Brattleboro rats [30]. Further, the regulation of circadian rhythms differs between Brattleboro and control Long-Evans rats. When the food-entrainable oscillator (FEO) and light-entrainable oscillator (LEO) are 3. King DP, Zhao Y, Sangoram AM et al. Cell 16, 641–653 (1997).


tor (FEO) and light-entrainable oscillator (LEO) are consistent with the hypothesis that VP acts as a modulatory signal which amplifies other output signals from SCN. It remains to be determined how these VP-ergic cells act in concert with other SCN signals to regulate the phase of circadian rhythms in target cells in the rest of the brain and body.

Conclusion

The overall peptidergic organization of the SCN in the CLOCK mutant mouse is the same as that in the wild type mouse, and similar to that described in rat and hamster. Only one neuropeptide, VP, shows decreased expression in the SCN of CLOCK mutant mouse, despite the presence of upstream E-boxes in other SCN peptides. Hence, it is likely that multiple regulatory domains regulate expression of SCN neuropeptides; these same elements may confer regional specificity in the control of VP and perhaps other peptides. Finally, the present findings are consistent with the hypothesis that VP acts as a modulatory signal which amplifies other output signals from SCN. It remains to be determined how these VP-ergic cells act in concert with other SCN signals to regulate the phase of circadian rhythms in target cells in the rest of the brain and body.

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