Title

Aging attenuates acquired heat tolerance and hypothalamic neurogenesis in rats

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ABSTRACT

This study investigated age-dependent changes in heat exposure-induced hypothalamic neurogenesis and acquired heat tolerance in rats. We previously reported that neuronal progenitor cell proliferation and neural differentiation are enhanced in the hypothalamus of long-term heat-acclimated (HA) rats. Male Wistar rats, 5 weeks (Young), 10–11 months (Adult), or 22–25 months (Old) old, were subjected to an ambient temperature of 32°C for 40–50 days (HA rats). Rats underwent a heat tolerance test. In HA rats, increases in abdominal temperature (T_{ab}) in the Young, Adult, and Old groups were significantly smaller than those in their respective controls (CNs). However, increase in T_{ab} of HA rats became greater with advancing age. The number of hypothalamic bromodeoxyuridine (BrdU)-immunopositive cells double stained with

a mature neuron marker, neuronal nuclei (NeuN), of HA rats was significantly higher in the Young group than that in the CN group. In Young HA, BrdU/NeuN-immunopositive cells of the preoptic area/anterior hypothalamus appeared to be the highest among regions examined. A large number of newborn neuron was also located in the ventromedial and dorsomedial nuclei, and posterior hypothalamic area, whereas heat exposure did not increase this in the Adult and Old groups. Aging may interfere with heat exposure-induced hypothalamic neurogenesis and acquired heat tolerance in rats.

INDEXING TERMS: Thermoregulation, Aging, Heat acclimation, BrdU, Hypothalamus

Chronic exposure to moderate heat provides heat acclimation that comprises thermoregulatory functions to acute heating. Heat-acclimated animals manifest lower core temperatures by reinforced heat dissipation and delayed increases in core temperatures when exposed to intense heat (Wyndham et al., 1976, Garrett et al., 2009, Sawka et al., 2011). In rodents, two phenotypes of heat acclimation, namely short-term heat acclimation (SHA) and long-term heat acclimation (HA), are known and depend on the duration of heat exposure (Horowitz et al., 1998). The characteristics of SHA and HA are clearly distinguished, e.g., functional changes in SHA are rapidly lost after heat exposure ends, whereas those in HA are stable and sustained (Garrett et al., 2009; Williams et al., 1967). In the later phase, changes in the ratio of thermosensitive to

insensitive preoptic/anterior hypothalamus (PO/AH) neurons after the improvement of HA (Pierau et al., 1994) confirm the existence of neuronal plasticity in the hypothalamic regions responsible for thermoregulatory integration during heat acclimation. Furthermore, several investigations have been conducted in PO/AH from various perspectives regarding gene expression profiles (Schwimmer et al., 2006), and morphological changes (Armstrong and Stoppani, 2002) have been reported in the synaptic structures of HA rats. Thus, persistent functional and/or morphological changes in the hypothalamus may be expected with HA.

It is now widely accepted that the hypothalamus can generate newborn neurons (Kokoeva et al., 2005; Migaud et al., 2010; Xu et al., 2005) as well as the

subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG; Gould et al., 1993, Gage, 2000; Magavi et al., 2000; Pencea et al., 2001). The number of newly generated neurons in the hypothalamus is much lower than the number of neurons added to the olfactory bulbs and DG. However, newly generated neurons in the hypothalamus appear to receive synaptic input indicative of their electrical integration in local hypothalamic circuits (Robins et al., 2013) and have a significant role in the acquisition and maintenance of brain function, e.g., hypothalamic newborn neurons are involved in the energy balance system in mice (Kokoeva et al., 2005). Moreover, the influence of aging on decremental neurogenesis in several brain regions has been studied in detail (Hattiangady and Shetty, 2008; Kuhn et al., 1996; Lazarov et al., 2010). We previously

reported that constant exposure to moderate heat facilitated progenitor cell proliferation and neuronal differentiation in the hypothalamus. Moreover, hypothalamic newborn neurons induced by heat exposure have been shown to become incorporated into neural circuits. Newborn neurons in the hypothalamus of HA rats may participate in the acquired HA (Matsuzaki et al., 2009). On the other hand, the ability to thermoregulate in response to acute heating diminishes with age, e.g., aging decreases the capacity to sweat, reduces cardiac output and stroke volume, and reduces skin blood flow in human subjects exposed to heat environments (Armstrong and Kenney, 1993; Kenney and Fels, 2003; Minson et al., 1998). One implication of these results is that central and sympathetic neural responses to direct passive heating attenuate with aging. However, to our knowledge, little is known if and about how

aging influences the ability to improve heat tolerance.

On the basis of these findings, we hypothesized that aging of animals influences the acquisition of HA and heat exposure-induced hypothalamic neurogenesis in rats. To test this hypothesis, we assessed the acquired HA in rats of different ages. Subsequently, progenitor cell proliferation and differentiation in the hypothalamus were studied.

MATERIALS AND METHODS

All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of the Shimane University Faculty of Medicine, which were compiled

from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science.

Experimental schedule

Thirty-six male Wistar rats were obtained from Japan SLC (Shizuoka, Japan) and used for the study. Rats, 5 weeks (Young), 10–11 months (Adult), and 22–25 months (Old) old, were individually housed in transparent plastic cages (width, 270 mm; length, 440 mm; height, 187 mm) with wood chippings and initially maintained at an ambient temperature (T_a) of 24.0 ± 0.1°C and relative humidity of 45 ± 5% under a 12:12-h light–dark cycle. At first, the rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and a temperature transmitter (TA10TA-F40; Data Sciences, St. Paul,

MN, USA) was implanted in their intraperitoneal cavity. The rats were allowed to recover from surgery for at least 2 weeks prior to data collection. All rats were housed individually to avoid crosstalk of radio telemetry. After the recovery period, the rats for HA were subjected to a constant T_a of 32.0 ± 0.2°C and relative humidity of 40 ± 10%, whereas the control (CN) rats were continuously kept at 24.0 ± 0.1°C. To detect newly generated cells, bromodeoxyuridine (BrdU; Sigma, St Louis, MO, USA) was dissolved in saline (10 mg/ml) and daily injected into the abdominal cavity of the rats (50 mg/kg/day) for 5 consecutive days after starting heat exposure. On the 40^{th}–50^{th} day after the start of the heat exposure period, all the animals were subjected to a heat tolerance test.

Heat tolerance test

After the end of the heat exposure period, abdominal temperature (T_{ab}) of HA-rats was unstable immediately after long-term heat exposure. Therefore, all the rats were maintained at T_a of 24°C for 48 h with food and water ad libitum to stabilize T_{ab}. Following this, the CN and HA rats were subjected to a 180-min thermal gradient that was increased by 0.7–0.8°C every 10 min from 24–36°C, as shown in Fig. 1A. When the rats were subjected to the heat tolerance test, the abdominal temperature (T_{ab}) of the rats in each group was measured by telemetry (Dataquest; Data Sciences International). Food and water were removed during the heat tolerance test.

Immunohistochemistry

After the heat tolerance test, pentobarbital sodium (50 mg/kg, i.p.) was used to anesthetize the rats and was transcardially perfused with ice cold 4% Paraformaldehyde phosphate buffer solution (Wako, Tokyo, Japan) preceded by a saline perfusion. The brain was removed, fixed overnight at 4°C in 4% formaldehyde phosphate buffer solution, and immersed in 20% (w/v) sucrose solution. A cryostat was used to prepare brain sections (40-µm thickness), which were collected as free-floating sections, of all the rats. For the detection of BrdU incorporation, the brain sections were incubated in 50% formamide/2× standard sodium citrate for 2 h at 65°C, incubated in 2 N HCl for 30 min at 37°C, rinsed in 100 mM boric acid (pH 8.5) for 10 min at 25°C, and washed with 0.25% Triton X-100 in Tris-buffered saline (pH 7.4). For multiplex immunoassaying, coronal sections were incubated with several primary

antibodies for 12 h at 4°C. The primary antibodies used in this study were monoclonal rat anti-BrdU IgG (1:10; Oxford Biotechnology Cat# OBT00030G RRID:AB_2314038), monoclonal mouse anti-neuronal nuclei (NeuN) IgG (1:500; Chemicon, Cat# mAbA60 RRID:AB_2314891), polyclonal rabbit anti-doublecortin (Dcx) IgG (1:200; Abcam Cat# ab18723 RRID:AB_732011), polyclonal goat anti-adenomatous polyposis coli (APC) IgG (1:100; Santa Cruz Biotechnology Cat# sc-896 RRID:AB_2057493), and polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) IgG (1:100; Sigma-Aldrich Cat# G9269 RRID:AB_477035). To identify the localization of BrdU-immunopositive (BrdU+) cells colabeled with NeuN, Dcx, APC, and GFAP, Alexa Fluor 633 anti-rat IgG with Alexa Fluor 488 anti-mouse IgG, Alexa Fluor 488 anti-rabbit IgG, and Alexa Fluor 488 anti-goat IgG (1:500; Molecular Probes

Cat# A21094 RRID:AB_141553, Cat# A21094 RRID:AB_14155, Cat# A11008 RRID:AB_143165, Cat# A11029 RRID:AB_138404) were used as the secondary antibodies. To establish the hypothalamic area, BrdU-labeled sections were counterstained with DAPI (Dojindo, Osaka, Japan). After staining, sections were mounted on glass slides and covered with 80% glycerol.

Cell counting

A confocal microscope (Olympus FV-300; Olympus, Tokyo, Japan) and imaging software (Olympus Fluoview; Olympus) were used to visualize all sections under 20× or 40× magnifications. A Cy5 filter was used to observe the BrdU+ cells, and an FITC filter was used to detect the other colabeled cells. For the hypothalamic area, brain

sections (between −0.26 and −4.80 mm from the bregma) were obtained according to the Paxinos and Watson atlas (Paxinos and Watson, 1998). Because constitutive birth of new neurons has been unambiguously demonstrated within SGZ of DG, BrdU+ cells of this area were also inspected. For hippocampal area, brain sections between −2.12 mm and −5.30 mm from the bregma were collected. BrdU+ cells were counted in 12 sections per animal, as described previously (Matsuzaki et al., 2009). Because the BrdU-labeled nuclei were counted at one-sixth interval sections, the possibility of counting split cells on different sections was minimized to less than 10%, according to the equation of Abercrombie (Guillery et al., 1997). Individual BrdU+ cells stained with NeuN, GFAP, or Dcx were also counted. All cells were counted blind with regard to the rat status.

Survival and migration of hypothalamic newborn cells at the onset of heating

An additional study was conducted to investigate the survival and migration of hypothalamic newborn cells. Eighteen rats were used for additional experiment. The rats were exposed to a constant T_a of 32°C for only 6 consecutive days (SHA rats; n = 3), whereas CN rats (SCN; n = 3) were kept at a T_a of 24°C. BrdU was daily injected into the rats' abdominal cavities (50 mg/kg/day) for the first 5 consecutive days of heat exposure. On the 6^{th} day after starting heat exposure, the rats were anesthetized and the brain was used for immunohistochemistry as described above.

Data quantification and statistical analysis

The results are presented as the mean ± S.E.M. The parameters obtained were analyzed by two-way (age and heat exposure) analysis of variance (ANOVA) and effects of age on parameter were analyzed by one-way ANOVA with Tukey's post hoc test. Statistical analyses were performed with SPSS (version 18.0, IBM, RRID:rid_000042) software. A P value <0.05 was considered to indicate statistical significance.

RESULTS

The mean T_{ab} values during the daily light and dark phases in the Young, Adult, and Old groups are summarized in Table 1. Before heat exposure, T_{ab} of all the rats at T_a of 24°C did not differ. Constant exposure to moderate heat significantly increased T_{ab} both in the light [Young: $F_{(2, 30)} = 45.6$, $P < 0.001$; Adult: $F_{(2, 30)} = 39.9$, $P < 0.001$; Old:

$F_{(2, 30)}$ = 36.2, P < 0.001, two-way ANOVA with Tukey's post hoc test] and dark [Young: $F_{(2, 30)}$ = 43.2, P < 0.001; Adult: $F_{(2, 30)}$ = 42.6, P < 0.001; $F_{(2, 30)}$ = 42.2, Old: P < 0.001, two-way ANOVA with Tukey's post hoc test] phases.

Acquired heat tolerance

During heat tolerance test, T_a was raised from 24°C to 36°C as shown in Fig. 1A. The mean T_{ab} values of all the groups during the heat tolerance test are shown in Fig. 1B (top). The T_{ab} values of the long-term heat-acclimated Young (Y-HA), Adult (A-HA), and Old (O-HA) groups at 24°C were clearly lower than those of respective CNs. The increases in T_{ab} from the initial levels (ΔT_{ab}) are shown in Fig. 1B (bottom). In all the rats, ΔT_{ab} gradually increased with time. The increase in ΔT_{ab} values of Y-HA, A-HA,

and O-HA was slower over time compared with that in ΔT_{ab} values of their respective CNs. However, the increases in ΔT_{ab} in the HA groups became greater with advancing age (Fig. 1B bottom). The values obtained by subtracting the ΔT_{ab} values of Y-HA, A-HA, and O-HA from those of CNs at 180 min after starting the heat tolerance test are shown in Fig. 1C. The T_{ab} differences between the CN and HA groups at 180 min after commencing heat load were 1.18°C in the Young group, 0.69°C in the Adult group, and 0.48°C in the Old group (Fig. 1C). The T_{ab} difference of the Adult and Old groups was significantly smaller than that of the Young group [$F_{(2, 15)} = 97.88$, $P < 0.001$, one-way ANOVA with Tukey's post hoc test].

BrdU+ cells in the hypothalamus

In the CN rats, the number of BrdU+ cells gradually decreased with aging [$F_{(2, 15)}$ = 10.42, $P < 0.001$, one-way ANOVA with Tukey's post hoc test]. A large number of BrdU+ cells of the Y-HA group were widely dispersed in the hypothalamic parenchyma (Fig. 2A and B). However, heat exposure did not alter the number of hypothalamic BrdU+ cells in the Adult and Old groups (Fig. 2C and D). The number of BrdU+ cells in the Y-HA group was significantly greater than that in the Y-CN group, whereas in the Adult and Old groups; however, BrdU+ cells were at very low levels in the hypothalamus [Fig. 2G, $F_{(2, 30)}$ = 10.08, $P < 0.05$, two-way ANOVA with Tukey's post hoc test].

BrdU+ cells in SGZ

To investigate whether aging affects heat exposure-induced progenitor cell proliferation, we inspected BrdU+ cells in SGZ (Fig. 3A to F). In CNs, the number of BrdU+ cells of SGZ gradually decreased with aging [Fig. 3G, $F_{(2, 15)} = 7.42$, $P < 0.01$, one-way ANOVA with Tukey's post hoc test]. However, heat exposure did not alter the number of BrdU+ cells between each CN and HA rat in SGZ (Fig. 3G).

NeuN expression in the hypothalamic and hippocampal BrdU+ cells

In the Y-CN group, some BrdU and NeuN double-labeled cells (BrdU+/NeuN+ cells) were observed in the hypothalamic parenchyma (Fig. 4A). The percentage of BrdU+/NeuN+ cells to BrdU+ cells in the Y-CN group was 11.2%. In contrast, in the Y-HA group, a large number of BrdU+/NeuN+ cells were observed in the

hypothalamic parenchyma (Fig. 4B). The percentage of BrdU+/NeuN+ cells to BrdU+ cells in the Y-HA group was 34.2%. The number of BrdU+/NeuN+ cells in the Y-HA group was significantly higher than that in the Y-CN group [Fig 4F, $F_{(2, 30)} = 7.51$, $P < 0.01$, two-way ANOVA with Tukey's post hoc test]. However, there were very few BrdU+/NeuN+ cells in the hypothalamus of the Adult and Old groups for both the CNs and HA rats, and heat exposure did not change the number of BrdU+/NeuN+ cells in the Adult and Old groups (Fig. 4C, D and F). The ratios of BrdU+/NeuN+ double-positive cells to BrdU+ cells in the hypothalamic areas of the A-CN and O-CN rats were not significantly different from those in the hypothalamic areas of the A-HA and O-HA rats, e.g., 9.2% in the O-CN rats and 10.1% in the O-HA rats. In addition, BrdU+/NeuN+ cells of hippocampal sections were inspected. Almost (over 90%) of

BrdU+ cells were double-labeled with NeuN in the hippocampus of all sub groups. However, heat exposure did not affect the number of BrdU/NeuN+ in the Young, Adult and Old rats (data not shown).

Dcx expression in the hypothalamic BrdU+ cells

BrdU+ cells labeled with Dcx (BrdU+/Dcx+ cells) were widely dispersed in the hypothalamus of both the Y-CN and Y-HA groups (Fig. 5A and B). The total counts of hypothalamic BrdU+/Dcx+ cells for Y-HA rats were significantly higher than those for Y-CN rats [Fig. 5F, $F_{(2, 15)} = 4.62$, $P < 0.05$, two-way ANOVA with Tukey's post hoc test]. In the Adult and Old groups, however, BrdU+/Dcx+ cells were hardly expressed in the hypothalamus (Fig. 5C and D), and Dcx immunoreactivity was considerably

smaller than that of the Young group.

Glial marker expression in hypothalamic BrdU+ cells

BrdU+ cells were double labeled with an anti-APC antibody, an oligodendrocyte marker, in the hypothalamus. In all the subgroups, a small number of BrdU+ cells stained with APC (BrdU+/APC+ cells) were detectable in the hypothalamus. In the Y-HA and A-HA groups, heat exposure slightly increased the total number of BrdU+/APC+ cells relative to those in the respective CNs (Fig. 6A and B), but there was no significant difference. In the Old group, heat exposure hardly altered the number of BrdU+/APC+ cells in the hypothalamus (Fig. 6C). Moreover, brain sections were immunolabeled with an anti-BrdU antibody together with an anti-GFAP antibody,

an astrocyte marker. However, BrdU+ cells expressing GFAP (BrdU+/GFAP+ cells) were rarely detected in any subgroup (Fig. 6D-F).

Hypothalamic BrdU+ cells in SHA rats

The number of BrdU+ cells in the Young SHAs (Y-SHAs) was significantly higher than that in the CNs (Y-SCNs; $P < 0.05$). In the Y-SHA rats, a large number of BrdU+ cells was detected in the ependymal layer of the third ventricle (Fig. 7A). However, small numbers of BrdU+ cells in the SHA Adult (A-SHA) and SHA Old (O-SHA) groups were detected (Fig. 7B). Interestingly, the number of BrdU+ cells in the Y-SHA group decreased from approximately 200 to 120 cells in the hypothalamus of the Y-HA group, which indicated that approximately 60% of newborn cells in the young

hypothalamus survived more than 30 days. However, in aged rats, approximately 43% of BrdU+ cells in the Adult group and 11% in the Old group remained in the hypothalamus.

DISCUSSION

In the present study, we examined the effects of aging on acquired heat tolerance and heat exposure-induced hypothalamic neurogenesis in rats. Heat tolerance tests revealed that the magnitudes of increase in the T_{ab} values of the Y-HA, A-HA, and O-HA groups was significantly smaller than that in the T_{ab} values of the respective CN groups, suggesting that HA improved heat tolerance in the Young, Adult, and Old groups. However, the increases in the T_{ab} values of the HA rats became greater with advancing

age (Figs. 1). This result indicated that acquired heat tolerance was gradually prevented in an age-dependent manner. On the other hand, immunohistochemical analysis revealed that heat exposure increased the number of BrdU+ cells in the hypothalamus of the Y-HA rats, whereas heat exposure did not change the same in the A-HA and O-HA rats (Fig. 2). In addition, heat exposure increased the number of hypothalamic BrdU+ cells in the Y-HA rats, which expressed the neuronal markers NeuN and/or Dcx, whereas these cells tended to decrease in the A-HA and O-HA rats (Figs. 4 and 5). There is a possibility that heat exposure increased the proliferation of hypothalamic progenitor cells and neuronal differentiation in young rats but did not induce these effects in senescent rats. In contrast, a small number of hypothalamic BrdU+ cells were stained with APC, a marker of oligodendrocytes, and BrdU+ cells rarely expressed

GFAP, a marker of astrocytes (Fig. 6). These results suggest that proliferated progenitor cells following heat exposure do not show glial differentiation in the hypothalamus. Associated with BrdU immunostaining and phenotyping, we confirmed that heat tolerance test and breeding of 48 h at 24°C before the test does not significantly affect the number and phenotype of BrdU+ cells.

In particular, hypothalamic neuronal progenitor cells exist and proliferate in the ependymal layer of the third ventricle and migrate into the hypothalamic parenchyma where they differentiate into neurons (Xu et al., 2005). We have previously reported that neuronal progenitor cell proliferation in young rats was enhanced within 6 days after starting heat exposure in the ependymal layer of the third ventricle and that nearly

60% of newborn cells survived for at least 53 days in the hypothalamic parenchyma (Matsuzaki et al., 2009). Therefore, we additionally examined whether aging affects the survival and migration of newborn cells. The number of BrdU+ cells in the short-term heat-exposed rats decreased from more than 200 to approximately 120 (Fig. 2G and Fig. 7C). This result suggested that approximately 60% of hypothalamic newborn cells induced by heat exposure survived over 40 days. From this age onward, the rate of newborn cell survival remarkably declined to the very low cell counts found in the A-HA (43%) and O-HA (11%) rats, which suggests that aging deteriorates not only progenitor cell proliferation but also newborn cell survival and maturation in the hypothalamus. Moreover, in young group, the distribution of BrdU+ cells in the hypothalamus of the short term and long term heat-exposed rats was different. In short

term heat-exposed young rats, a large number of BrdU+ cells existed in the ependymal layer of the third ventricle, whereas most of the BrdU+ cells in the Y-HA rats were within the hypothalamic parenchyma (Fig. 2B and Fig. 7A). Although we did not directly measure proliferation and migration of BrdU+ cells, these results suggest that heat exposure increases the proliferation of neuronal progenitor cells in the ependymal layer of the third ventricle of young rats and proliferated cells then appear to migrate into the hypothalamic parenchyma. However, this difference in newborn cell distribution was not observed in older rats. As a prediction, this distributional alteration of hypothalamic newborn cells may be partially related to the changes in the expression level of Dcx protein, a developmentally regulated factor of newborn neurons. Dcx is typically associated with the migration of newborn neurons during the

development of the central nervous system (Brown et al., 2003). The expression level of Dcx protein is kept high during the migration of newborn neurons within certain areas of the adult mammalian brain. In this study, immunohistochemical analysis revealed clearly stained Dcx single-labeled cells in the hypothalamus of the Y-HA rats, whereas the number and immunoreactivity of Dcx was meager in the O-HA rats (Fig. 5A-D). Thus, age-dependent declines in Dcx protein expression may be at least partially related to the distributional changes of BrdU+ cells in the hypothalamus.

During the 40–50-day heat exposure period, the T_{ab} values in the Y-HA, A-HA, and O-HA rats were significantly higher than those in their respective CNs (Table 1). High temperatures physically facilitate biological reactions and then may increase cell

proliferation in the brain. Therefore, BrdU+ cells in SGZ, which has a strong proliferative potency of progenitor cells, were counted. However, heat exposure and the associated increase in core temperature had a minimum influence on cell proliferations in SGZ in all the subgroups (Fig. 3). These observations demonstrate that the rise in temperature of progenitor cells may not directly lead to the proliferation of progenitor cells.

In this study, we found that aging attenuated improvement of heat tolerance and hypothalamic neurogenesis in HA rats. However, aged rats improved heat tolerance despite the absence of neurogenesis in the hypothalamus. These observations suggest that the central mechanism of HA depends on not only hypothalamic neurogenesis but

also other functions such as the plasticity of existing neurons. In a previous study, Meiri et al (1991) have shown that aging retarded vasomotor response and reduced tolerance to heat stress when hypohydrated condition in rats. Associated with this, newly born neuron in PO/AH of Y-HA may be at least partially contribute to skin vasomotor activity in heat environment. Further studies are required to elucidate the central mechanism of HA and age-related reductions of heat exposure-induced hypothalamic neurogenesis.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: K.M. and O.S. Provide aged-rats: M.K. and T.I. Acquisition of data: K.M. Obtained funding: K.M. and O.S. Critical revision of the article for important intellectual content: O.S., M.K., T.I. T.H. and M.H. Study supervision: O.S., M.K., T.I., T.H. and M.H.

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Figure Legends

Table 1. Mean T_{ab} values of the Young, Adult, and Old rats. Before: a day before starting the heat exposure period. During: the last day of the heat exposure period. Values are the mean ± S.E.M (n = 6). Heat exposure significantly elevated T_{ab} in light and dark phase of all subgroups. *, significant difference between control (CN) and long-term heat-acclimated (HA) rats.

Figure 1. Heat tolerance test of long-term heat-acclimated (HA) rats. (A) T_a of the chamber in the heat tolerance test. (B) T_{ab} (top) and ΔT_{ab} (bottom) responses to gradient heat of Young (left), Adult (center), and Old (right) rats. The T_{ab} values of all rats were monitored by a telemetry system. When T_a was raised from 24°C to 36°C,

the increase in the T_{ab} values was slower for the Y-HA (closed circle), A-HA (closed triangle), and O-HA (closed square) rats than that for the Y-CN (open circle), A-CN (open triangle), and O-CN (open square) rats. However, the amount of increase in T_{ab} in the HA rats became greater with advancing age. Differences in T_{ab} between Young, Adult, and Old CN and HA rats were analyzed by two-way ANOVA. Values are the mean ± S.E.M. (C) T_{ab} difference at 180 min after starting the heat tolerance test between the Young, Adult, and Old CN and HA rats. The T_{ab} difference decreased in an age-dependent manner. #, significant difference between Young and Adult. *, significant difference between Adult and Old. Values are the mean ± S.E.M (n = 6).

Figure 2. BrdU+ cells in the hypothalamus. BrdU+ cells in the hypothalamus of Y-CN

(A), Y-HA (B), O-CN (C) and O-HA (D). Scale bars, 100 μm. BrdU+ cells in Adult rats are not shown. The density of hypothalamic BrdU+ cells is much greater in the Y-HA rats than that in the O-HA rats. (E and F) Bottom panels show magnified views of boxed regions from the Y-CN (E) and Y-HA (F). Scale bar, 10 μm. (G) Box and whisker plot of BrdU+ cells in the hypothalamus of the CN (white bars) and HA (gray bars) rats (n = 6). *, significant difference between Y-CN and Y-HA. (H) Over view of hypothalamic section. Red box shows immuno-stained area of (A), (B), (C) and (D). ac, anterior comm. ox, optic chiasm.

Figure 3. BrdU+ cells in SGZ. BrdU-labeled sections of the hippocampus in Y-CN (A), Y-HA (B), O-CN (C) and O-HA (D). Scale bar, 100 μm. DG, dentate gyrus. BrdU+

cells in the Adult rats are not shown. Bottom panels show magnified views of boxed regions from the Y-CN (E) and Y-HA (F). Scale bar, 10 μm. (G) Box and whisker plot of BrdU+ cells in SGZ of CN (white bars) and HA (gray bars) rats (n = 6). Heat exposure did not alter the numbers of BrdU+ cells in SGZ. (H) Overview of DG. Red box shows immuno-stained area of (A), (B), (C) and (D).

Figure 4. Colabeling of BrdU with the mature neuronal marker NeuN by confocal optical sectioning. BrdU- and NeuN-labeled sections of the hypothalamus in Y-CN (A), Y-HA (B), O-CN (C) and O-HA (D). Arrows denote double-labeled cells. 3V, third ventricle. Scale bar, 100 μm. BrdU+/NeuN+ cells in the Adult rats are not shown. (E) Confocal reconstruction of the area boxed in (B). Left, NeuN; center, BrdU; right,

merge. Scale bar, 10 μm. (F) Box and whisker plot of BrdU+/NeuN+ cells in the hypothalamus of CN (white bars) and HA (gray bars) rats belonging to the different age groups. Heat exposure considerably enhanced the numbers of newly born neurons in the hypothalamus of the Young rats but tended to decrease the same in the Adult and Old rats. *, significant difference between Y-CN and Y-HA.

Figure 5. Colabeling of BrdU with the immature neuron maker Dcx by confocal optical sectioning. BrdU- and Dcx-labeled sections of the hypothalamus in the Y-CN (A), Y-HA (B), O-CN (C) and O-HA (D). BrdU+/Dcx+ cells in the Adult rats are not shown. Arrows denote double-labeled cells. 3V, third ventricle. Scale bar, 100 μm. (E) Confocal reconstruction of the area boxed in (B). Left, Dcx; middle, BrdU; right,

merge. Scale bar, 10 μm. (F) Box and whisker plot of BrdU+/Dcx+ cells in the hypothalamus of Young, Adult, and Old CN (white bars) and HA (gray bars) rats (n = 6). *, significant difference between Y-CN and Y-HA.

Figure 6. The number of colabeled cells of BrdU with glial markers. BrdU- and APC-labeled sections of the hypothalamus in the Y-HA (A) and O-HA (B). Scale bar, 100 μm. (C) Box and whisker plot of BrdU+ cells colabeled with APC in the hypothalamus (n=6). Heat exposure did not alter the number of BrdU+/APC+ cells in the hypothalamus. BrdU- and GFAP-labeled sections of the hypothalamus in the Y-HA (D) and O-HA (E). 3V, third ventricle. Scale bar, 100 μm. (F) Box and whisker plot of BrdU+ cells double labeled with GFAP in the hypothalamus. BrdU+/GFAP+ cells are

hardly detected in the hypothalamus of all subgroups (n = 6). Heat exposure did not change the numbers of BrdU+ cells double stained with each glial marker in the hypothalamus.

Figure 7. BrdU+ cells in the hypothalamus of short-term heat-acclimated (SHA) rats. BrdU-labeled sections in the hypothalamus of Y-SHA (A) and O-SHA (B). A large number of BrdU+ cells were observed in the ependymal layer of the third ventricle of the Y-SHA rats, whereas very few were found in the O-SHA rats. 3V, third ventricle; scale bar, 100 μm. (C) Box and whisker plot of BrdU+ cells in the hypothalamus of SCN (white bars) and SHA (gray bars) rats (n = 3). *, significant difference between Y-SCN and Y-SHA.

Table 1. Table of Primary Antibodies Used

Antigen	Description of Immunogen	Source, Host Species, Cat. #, Clone or Lot#, RRID	Concentration Used
Bromodeoxyuridin (BrdU)	Bromodeoxyuridine	Oxford Biotechnology, rat monoclonal, Cat# OBT0030G, RRID:AB_2314038	10 umol/l (IHC)
Neuronal Nuclei (NeuN)	Purified neuronal nuclei from mouse brain	Chemicon, mouse monoclonal, Cat# MAB377, RRID:AB_2314891	1 ug/ml (IHC)
Adenomatous polyposis Coli (APC)	Affinity purified rabbit polyclonal antibody raised against the C-terminus of APC of human origin	SANTA CRUZ, rabbit polyclonal, Cat# sc-896 (C-20), RRID:AB_2057493	0.1ug/ul (IHC)
Glial Fibrillary Acidic Protein (GFAP)	Purified glial fibrillary acidic protein from human brain.	Sigma-Aldrich, rabbit polyclonal, Cat# G9269, RRID:AB_477035	0.1ug/ul (IHC)
Doublecortin (Dcx)	Affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of Doublecortin of human origin.	Abcam, rabbit polyclonal, Cat# ab18723, RRID:AB_732011	0.1ug/ul (IHC)

Antibody Characterization
Please see Table 1 for a list of all antibodies used.

Anti BrdU antibody recognizes bromodeoxyuridine (BrdU). This antibody reacts with BrdU incorporated into single stranded DNA, attached to a protein carrier and free BrdU. This antibody does not cross react with thymidine or iododeoxyuridine.

Anti-NeuN monoclonal antibody reacts with most neuronal cell types thought the nervous system of mice including cerebellum, cerebral cortex, hippocampus, thalamus, spinal cord and neurons in the peripheral nervous system including dorsal root ganglia. The immunohistochemical staining is primarily in the nucleus of the neurons with lighter staining in

the cytoplasm.

Anti-APC antibody (C-20) recognizes the C- terminus of APC. The adenomatous polyposis syndromes, familial adenomatous polyposis (FAP) and Gardner's syndrome (GS), are characterized by numerous adenomatous polyps throughout the entire colon. These polyps invariably progress to colon cancer in addition to other extracolonic manifestations. The cloning of the APC gene revealed a ubiquitously expressed protein, 2,843 amino acids in length, which is frequently mutated in patients suffering from FAP and GS. APC has been found to be associated with structural components of intracellular junctions.

Anti GFAP antibody reacts specifically with GFAP in immunoblotting assays and labels astrocytes, Bergmann glia cells and chondrocytes of elastic cartilage in immunohistochemical staining. The antibody reacts with glial specific antigen in frozen or alcohol-fixed tissue sections.

Anti Dcx Antibody is a rabbit polyclonal IgG. This antibody reacts with Dcx, a microtubule-associated protein expressed by neuronal precursor cells and immature neurons in embryonic and adult cortical structures, of mouse, rat and human.

Table 1

	T_{ab} (°C)							
	Before				During			
	CN		HA		CN		HA	
	Light phase	Dark phase	Light phase	Dark phase	Light phase	Dark phase	Light phase	Dark phase
Young	37.24 ± 0.06	37.89 ± 0.05	37.25 ± 0.07	37.84 ± 0.05	37.29 ± 0.02	37.80 ± 0.04	37.76 ± 0.09	38.61 ± 0.12*
Adult	37.19 ± 0.07	37.79 ± 0.09	37.21 ± 0.06	37.80 ± 0.08	37.34 ± 0.03	37.75 ± 0.04	37.82 ± 0.12	38.63 ± 0.08*
Old	37.20 ± 0.08	37.84 ± 0.07	37.20 ± 0.05	37.82 ± 0.07	37.32 ± 0.04	37.72 ± 0.03	37.91 ± 0.11	38.74 ± 0.09*

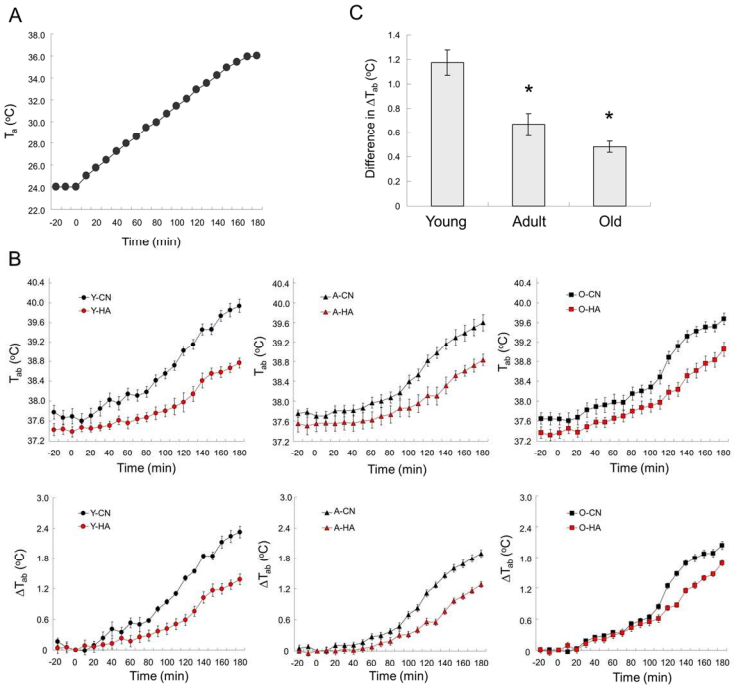

Fig.1

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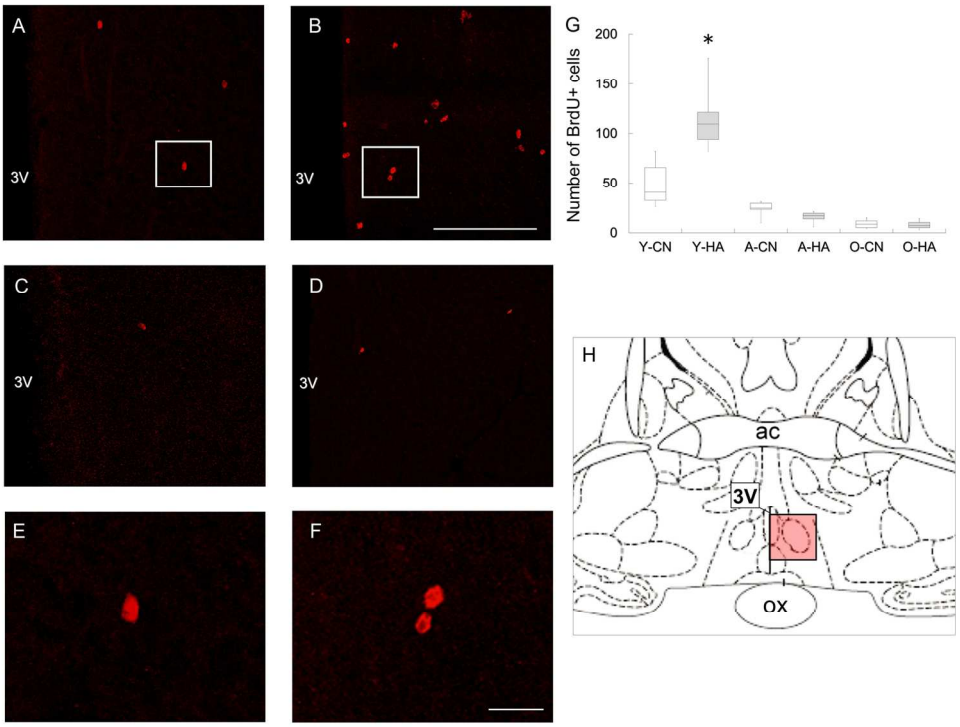

Fig.2

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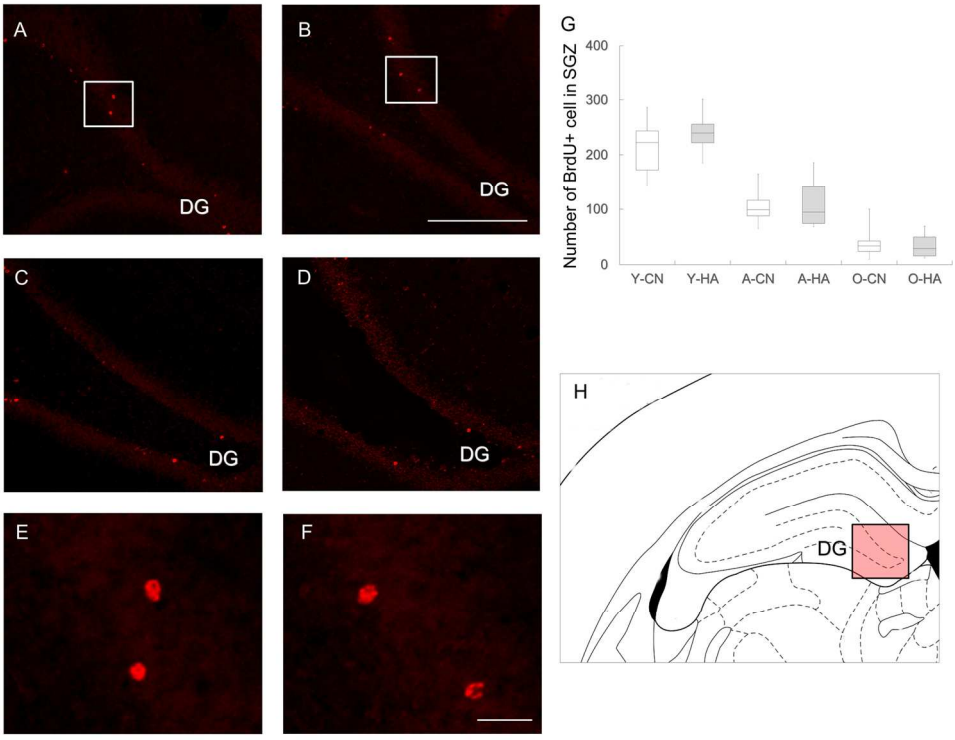

Fig.3

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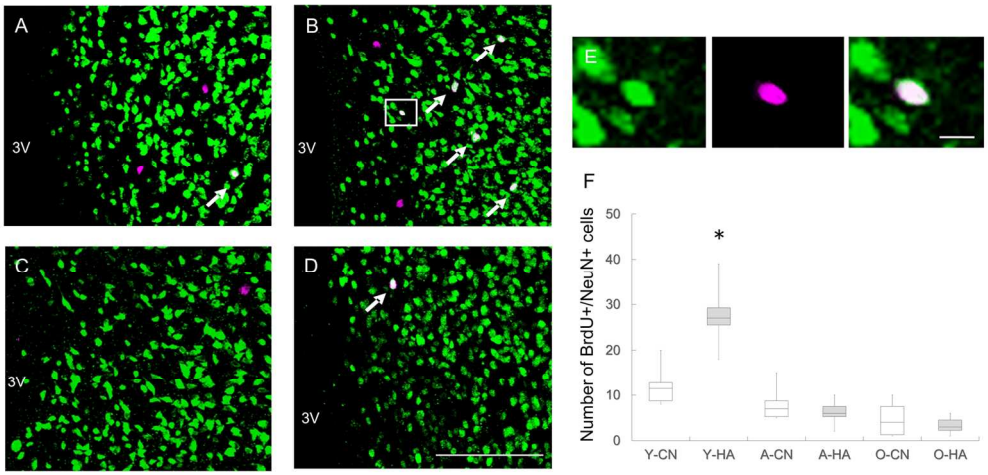

Fig.4

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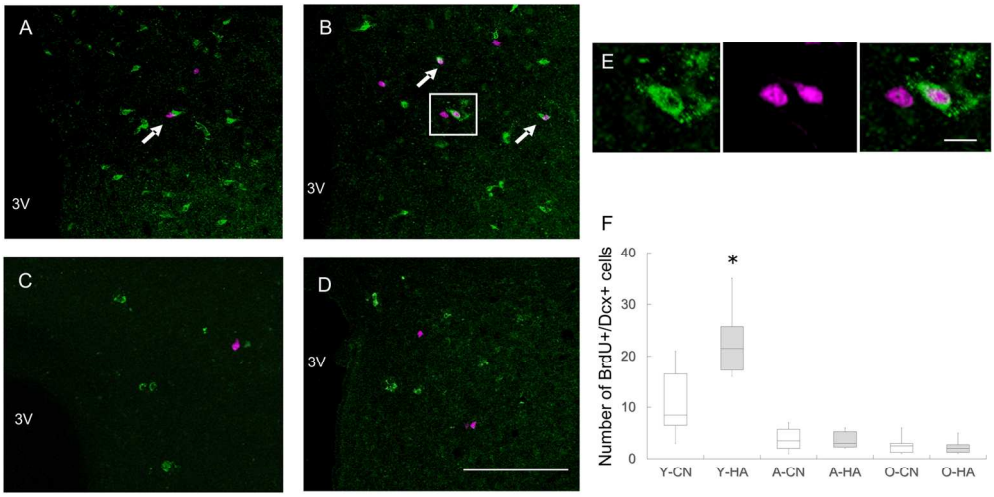

Fig. 5

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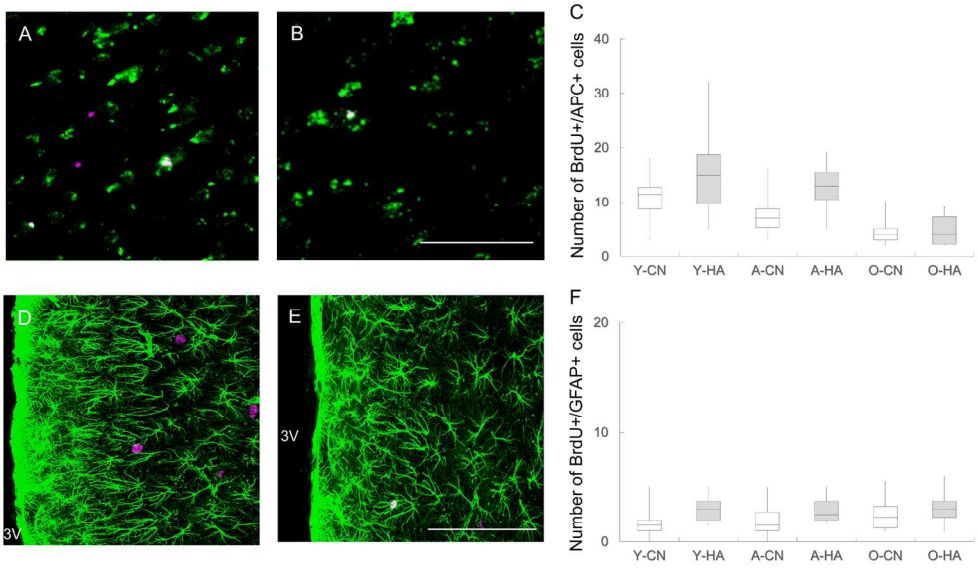

Fig. 6

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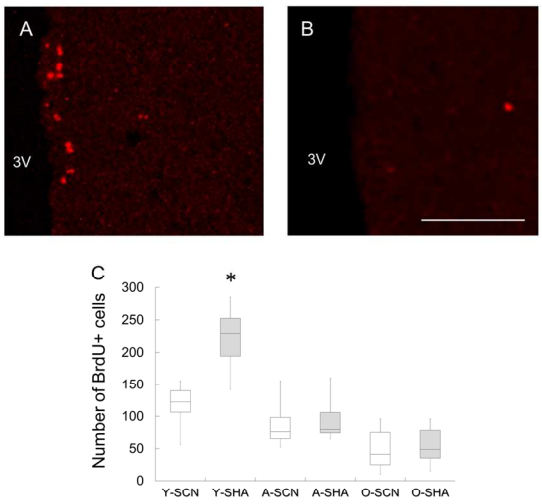

Fig. 7

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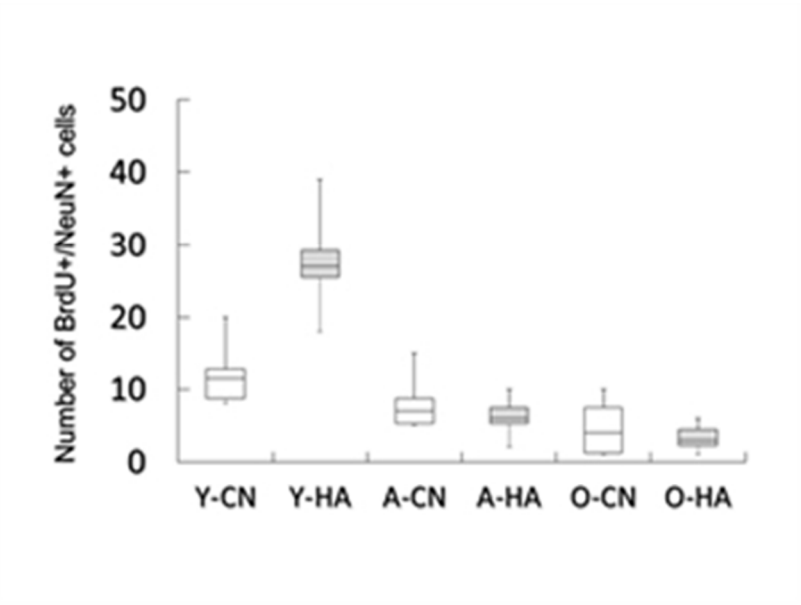
In heat tolerance test, increase in abdominal temperature of heat-acclimated rats became greater with advancing age. Immunohistochemical analysis showed that the number of hypothalamic BrdU-immunopositive cells of HA rats was significantly higher in the young rats than that of the control group, whereas heat exposure did not increase this in the elder rats. Aging may interfere with heat exposure-induced hypothalamic progenitor proliferation and improvement of heat tolerance.



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