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Melatonin inhibits neuronal dysfunction-associated with neuroinflammation by atopic psychological stress in NC/Nga atopic-like mouse models

Running title: Melatonin inhibits neuronal damage by atopic stress

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Abstract

Atopic dermatitis (AD), also known as atopic eczema, is chronic pruritic skin disease. AD can increase psychological stress as well, increasing glucocorticoid release and exacerbating the associated symptoms. Chronic glucocorticoid elevation disturbs neuroendocrine signaling, and can induce neuroinflammation, neurotoxicity, and cognitive impairment; however, it is unclear if AD-related psychological stress elevates glucocorticoids enough to cause neuronal damage. Therefore, we assessed the effects of AD-induced stress in a mouse AD model. AD-related psychological stress increased astroglial and microglial activation, neuroinflammatory cytokine expression, and markers of neuronal loss. Notably, melatonin administration inhibited the development of skin lesions, scratching behavior, and serum IgE levels in the model mice, and additionally caused a significant reduction in corticotropin-releasing hormone responsiveness, and a significant reduction in neuronal damage. Finally, we produced similar results in a corticosterone-induced AD-like skin model. This is the first study to demonstrate that AD-related psychological stress increases neuroendocrine dysfunction, exacerbates neuroinflammation, and potentially accelerates other neurodegenerative disease states.

Key words: atopy; hippocampus; melatonin; corticotropin-releasing hormone; glucocorticoids; neuronal damage

1. Introduction

Atopic dermatitis (AD), or atopic or hyperallergic eczema, is a chronic, relapsing inflammatory skin disease characterized by eczematous skin lesions, xerosis, lichenification, and severe pruritus ¹⁻². AD affects 10–20% of children and approximately 2–3% of adults worldwide ². The unpredictable flare-ups, intense and almost painful itching, and anxiety surrounding the potential for scarring all impose an immense psychological burden on those with AD ². Although not fully elucidated, the pathogenesis of AD is thought to involve dysregulation of the immune system; excessive Th2-derived cytokine release, hypersecretion of IgE, and eosinophilia have been found in patients with AD ³. Notably, stress can induce flare-ups or worsen AD symptoms. Patients with AD respond to psychosocial stress with blunted hypothalamic-pituitary-adrenal (HPA) axis responses and excessive sympathetic adrenomedullary responses ⁴. Thus, a psychoneuroimmune mechanism whereby stress leads to allergic inflammation may underlie its AD-aggravating effects ⁴⁻⁵. One study found the blunted HPA axis response to a stressor to be accompanied by atopy-relevant immunological changes ⁴⁻⁵. Furthermore, stress management psychotherapy and relaxation training can reduce itching and improve various AD symptoms ⁶.

Brain and skin communicate in both directions through the immune, the neuroendocrine, and steroidogenic system, stress effects on skin disease can be mediated through this system⁷⁻⁸. The HPA axis is a key neuroendocrine mediator of physiological responses to both psychological and physiological stressors ⁹⁻¹⁰. Corticotropin-releasing hormone (CRH) secreted from the hypothalamus stimulates the release of pituitary adrenocorticotrophic hormone (ACTH) into the blood; ACTH then stimulates glucocorticoid (cortisol in humans, corticosterone [CORT] in rodents) release from the adrenal cortex ^{11-12 13}. HPA activity is regulated by several negative feedback loops ¹³. Glucocorticoids act on many physiological systems to allow the body to react to a stressor, but their long-term chronic

elevation impairs or damages those target systems¹³⁻¹⁵. For example, Lupien et al. found that high levels of CORT in the rodent central nervous system (CNS) increased neuronal damage, decreased hippocampal volume, and caused memory deficits in rodents. Thune et al. demonstrated that exogenous glucocorticoids can adversely affect both hippocampal morphology and cognitive function in humans by inhibiting neurogenesis and/or increasing cell death¹⁶⁻¹⁹. Overall, a preponderance of evidence indicates that HPA axis hyperactivity induced by chronic stress is associated with neurotoxicity, cerebral atrophy, and cognitive impairment in the CNS, as well as impaired circadian rhythms of pituitary and adrenal secretions, blunted neuroendocrine responses to stress, and decreased sensitivity to glucocorticoid feedback¹⁹⁻²¹. Moreover, UVB exposures skin can activate the CRH with functional CRH-receptor 1 and related proopiomelanocortin (POMC) macromolecule, which is further processed to ACTH, which, after interaction with ACTH receptor, induces steroidogenesis with the final production of highly adaptable CORT²²⁻²³. Furthermore, UVB increases plasma α -melanocyte-stimulating hormone levels with systemic stimulation of epidermal melanocytes²². Moreover, UVB can activate POMC signaling in the arcuate nucleus of the hypothalamus and increases release of β -endorphin into the circulation²⁴. Not yet study, we assumed that HPA axis hyperactivity induced by UVB is accelerated the neuronal abnormalities. Recently, the inhibitory role of CRH on melatonin secretion in normal healthy men has been reported, suggesting an HPA axis-pineal gland interaction is also active.

The indolamine melatonin (N-acetyl-5-methoxytryptamine) is, under normal conditions, synthesized and released into the circulation by pineal gland and is responsible for chronibiotic effects, but also including among others the gastrointestinal tract, eyes, skin, and various immune tissues²⁵. Melatonin binds to two receptors, MT1 and MT2, although MT1 appears to be the predominant receptor, at least in skin. Melatonin has been shown both in

vitro and in vivo to be a powerful endogenous free radical scavenger and potent anti-inflammatory agent; it also stimulates antioxidative enzymes such as superoxide dismutase, glutathione peroxidase and glutathione reductase, protecting cell membranes from lipid peroxidation and neutralizing toxic radicals²⁶⁻²⁷. In addition, melatonin is a key regulator of circadian sleep-wake rhythms and neuroimmunological function²⁸. In allergic reactions, melatonin is an immunomodulatory agent. Recent evidence suggests that melatonin production decreases with age and in certain diseases, e.g., certain malignancies, Alzheimer's disease, and cardiovascular disease²⁹. This decrease in melatonin output has been linked to insomnia in older patients, to sleep disturbances in patients with Alzheimer's disease, and to a higher prevalence of cancer. Within atopic dermatitis, an index (severity) score of ≥ 48.7 predicts poor sleep efficiency, possibly as a result of lower nocturnal melatonin secretion, pruritus, and associated scratching, higher total serum IgE levels, and allergic sensitization³⁰. One experimental report demonstrated disturbed melatonin secretion in patients with atopic eczema, possibly due to the partially reduced sympathetic nervous activity that regulates melatonin secretion³⁰. Melatonin was also found to suppress the development of AD-like skin lesions in AD model (2,4-dinitrofluorobenzene-treated NC/Nga) mice by reducing total IgE in serum, and IL-4 and IFN- γ production by activated CD4⁺ T cells³¹. When the body or mind are stressed, melatonin binds adrenal cortical MT1 receptors to inhibit ACTH-stimulated cortisol production³². It was also found to inhibit dibutyril-cAMP-stimulated cortisol production, albeit through a cAMP-independent signaling pathway³³. Chronic melatonin exposure protects several regulatory components of the HPA axis from glucocorticoid-induced deterioration³⁴. However, the question of whether melatonin acts to reduce the psychological stress effects of AD remains.

Given the above data, we hypothesize that psychological stress can increase flare-ups of AD, which in turn increases the overall stress level further, creating a state of chronically

high glucocorticoid release. Such release would in itself produce dysfunction of melatonin, neuroinflammation, and CNS impairments; furthermore, it could worsen neuroinflammatory damage done by other neurodegenerative diseases such as Alzheimer's disease. Validated rodent models of atopic dermatitis are available; rodents are known to respond to high CORT and low melatonin similarly to humans, including demonstrable CNS toxicity, cognitive impairment, and accelerated atopy-relevant immunological changes^{10, 34-37}. Thus, we employed AD model mice to test the above hypothesis, including analyses of glucocorticoid status and the ability of melatonin to reduce AD-like skin lesions, CNS damage, and (neuro)inflammation. Because it is unknown whether atopy-induced psychological stress is associated with neuroinflammation in this model, we first confirmed the association in a CORT-induced AD-like model.

2. Materials and Methods

2.1 Chemical

2,4-dinitrochlorobenzene (*DNCB*; Lot. #MKBH8668V; $\geq 96.5\%$), mouse paraformaldehyde (PFA), 3,3-diaminobenzidine (DAB), sodium chloride, sucrose, ethanol, histo-mount medium, dimethylsulfoxide (DMSO) and phosphate buffered saline (PBS), melatonin, sesame oil, CORT, and sodium citrate buffer were purchased from Sigma–Aldrich (St. Louis, MO, USA). CytoScan™ lactate dehydrogenase (LDH)-cytotoxicity assay kit was obtained from Dojindo (Kumamoto, Japan). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin and fetal bovine serum (FBS), were purchased from Gibco (MD, USA). Clonetics PNGM SingleQuots was purchased from Lonza (MD, USA). Biotinylated horse anti-goat antibody, goat anti-rabbit antibody, rabbit anti-rat antibody, goat anti-mouse antibody, normal goat serum (NGS), normal horse serum (NHS), mouse on mouse detection kit, and VECTASTAIN Elite ABC Kit were purchased from Vector Laboratories (Burlingame, CA, USA). Mouse anti-

neuronal nuclear antigen (NeuN) antibody and fluoro-Jade® B were purchased from Millipore Bioscience Research (Bedford, MA, USA). Rabbit ionized calcium binding adaptor molecule 1 (Iba-1) antibody was purchased from Wako Pure (Tokyo, Japan). Goat anti-glia fibrillary acidic protein (GFAP), doublecortin (DCX) antibody, rabbit anti-cyclooxygenase (Cox)-2, and insulin-like growth factor-binding protein (IGFBP)-3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-inducible nitric oxide synthase (iNOS) antibody and NeuroTrace® 500/525 green fluorescent nissl stain were purchased from Thermo fisher (Waltham, MA, USA). Rabbit anti-pCreb antibody was purchased from cell signaling (Beverly, MA, USA). Mouse CORT EIA kit was purchased from Oxford (Oxford, MI, UK). Rabbit ACTH, and PhosphoTracer ERK1/2 (pT202/Y204)+p38 MAPK (pT180/Y182)+JNK1/2/3 (pT183/Y185) ELISA Kit were purchased from Abcam (Cambridge, UK). Rabbit melatonin receptor 1B antibody was purchased from Novus biologicals (Littleton, CO). Rabbit anti-CRHR1 and melatonin receptor 1A antibody and Mouse urocortin (UCN) and POMC ELISA kits were purchased from LSBio LifeSpan (Seattle, WA, USA). Mouse Cytokine Array C2000 was purchased from RayBio (Norcross, GA). Melatonin ELISA Kit was purchased from Aviva Systems Biology (San Diego, CA). All other reagents used were of guaranteed or analytical grade.

2.2 Animals

According to stenzel et al., female mice affects sex hormone and menstruation by CRH^{38, 39}. Thus, we selected that male NC/Nga mice (6 weeks, 18–20 g) were purchased from Orient Co., Ltd. (Tokyo, Japan) and maintained under temperature- and light-controlled conditions (20–23 °C, 12 h light/12 h dark cycle) with food and water provided ad libitum. All animals were acclimatized for 7 days prior to drug administration. The institutional animal care committee of Korea Institute of Oriental Medicine (KIOM) approved the experimental

protocol KIOM-16-015 and KIOM-16-112) and performed according to the guidelines of the Animal Care and Use Committee at KIOM.

2.3 DNCB treatments in mouse model

Briefly, for each mouse the dorsal fur was removed from an area of approximately 8 cm², and the mouse assigned to one of 5 groups (n = 8 per group). We fixed time day from P.M 15:30 to 18:30 for DNCB treatments. A sensitizing solution was applied to the dorsal skin for 14 consecutive days as follows: (1) vehicle (3:1 mixture of acetone and olive oil) control; (2) 0.5% DNCB solution; (3) 1% DNCB; (4) 2% DNCB; (5) 2% DNCB + 1% dexamethasone (DNCB-positive control, same vehicle). After sensitization, a 0.3% DNCB solution was applied to the dorsal skin of the mouse 3 times a week for 7 weeks. The skin must be gently clean by 70% Et-OH fabrics before repeat DNCB treatments. The animals were sacrificed at 7 weeks after sensitization with DNCB (See fig. 1 for a timeline).

2.4 DNCB treatments with melatonin in mouse model

The mice were assigned to 4 groups (n = 6 per group). AD-like skin lesions were induced by applying DNCB to the dorsal skin. We fixed time day from P.M 17:30 to 18:30 for DNCB treatments. After the 7-day sensitization period, the DNCB solution was applied to the dorsal skin of the mice seven times per week for an additional 4 weeks. The mice were assigned to 4 treatment groups: (1) vehicle (topical application of vehicle solution on the dorsal skin plus 5% DMSO in normal saline intraorally), (2) DNCB group (topical application of DNCB solution on the dorsal skin plus 5% DMSO in normal saline intraorally), (3) melatonin group (topical application of DNCB on the dorsal skin plus 20 mg/kg melatonin in 5% DMSO in normal saline intraorally), and (4) melatonin-only group (topical application of vehicle solution on the dorsal skin plus 20 mg/kg melatonin in 5% DMSO in normal saline intraorally). After

sensitization, a 0.3% DNCB solution was applied to the dorsal skin of the mouse 3 times a week for 4 weeks. The skin must be gently clean by 70% Et-OH fabrics before repeat DNCB treatments. The animals were sacrificed at 7 weeks after sensitization with DNCB (See fig. 1 for a timeline).

2.5 CORT treatments with melatonin in mouse model

The mice were assigned to 4 groups (n = 6 per group). We fixed time day from P.M 17:30 to 18:30 for CORT treatments and A.M 08:30 to 09:30 for melatonin treatments. The mice were assigned to 4 treatment groups: (1) vehicle (sesame oil subcutaneously plus 5% DMSO in normal saline intraperitoneal), (2) CORT group (20 mg/kg CORT in sesame oil subcutaneously plus 5% DMSO in normal saline intraperitoneal), (3) CORT and melatonin group (20 mg/kg CORT in sesame oil subcutaneously plus 20 mg/kg melatonin in 5% DMSO in normal saline intraperitoneal), and (4) melatonin-only group (sesame oil subcutaneously plus 20 mg/kg melatonin in 5% DMSO in normal saline intraorally); See fig. 1 for a timeline).

2.6 Scratching behavior, clinical score, and hematoxylin and eosin staining

Scratching behavior, clinical skin severity scores and were assessed, and hematoxylin and eosin staining was performed, according to previously published methods^{40, 41}. Briefly, mice were placed individually in clear plastic cages and their behavior was monitored for 30 min, 7 weeks after sensitization. Scratching of the rostral back and biting of the caudal back were observed; scratching movements by the hind paw were defined as a scratching bout that ended when the mice either licked its hind paw or placed its hind paw back on the floor, and a series of one or more biting movements were counted as one episode that ended when the rat returned to the straightforward position. Then, for evaluation of clinical skin severity scores, the five signs of skin lesions were (1) pruritus/itching, (2) erythema/hemorrhage, (3) edema,

(4) excoriation/erosion, and (5) scaling/dryness. The above-mentioned symptoms were graded as follows: 0 (no symptoms), 1 (mild), 2 (moderate), and 3 (severe).

2.7 Modified Y-maze test

The experiment was performed on day 55 of DNCB treatment. It is well known that spontaneous alternation is a measure of spatial working memory. The Y-maze can be used as a measure of memory and latency time. Therefore, spontaneous alternation was assessed using a Y-maze composed of three equally spaced arms (120°, 40 cm long and 3 cm wide with walls 12 cm high). The floor of each arm is made of Perspex and is 5 cm wide. Each mouse was placed in one of the arm compartments and was allowed to move freely until its tail completely entered another arm. The sequence of arm entries is manually recorded, the arms being labelled A, B, or C. An alternation is defined as entry into all three arms consecutively. That is, if the animal makes the arm entries ABC, CAB, BCA, and but not BAB, the animal is counted as having made 13 arm entries, 8 of which are spontaneous alternations. The number of maximum spontaneous alternations is calculated as the total number of arms entered minus two, and the percentage alternation is then calculated as $\{(\text{actual alternations}/\text{maximum alternations}) \times 100\}$. For each animal, Y-maze testing was carried out for a total of 40 entries. The apparatus was cleaned with 3% alcohol and allowed to dry between sessions. The total amount of entry time into arms was assessed as a parameter representing latency time.

2.8 Brain tissue preparation

Mice were immediately anesthetized and perfused transcardially with 0.05 M PBS, followed by cold 4% PFA in 0.1 M phosphate buffer. Brains were removed and post-fixed in 0.1 M phosphate buffer containing 4% PFA overnight at 4°C, and then immersed in a solution

containing 30% sucrose in 0.05 M PBS for cryoprotection. Serial 30- μ m-thick coronal sections were cut on a freezing microtome (Leica Instruments GmbH, Nussloch, Germany) and stored in cryoprotectant (25% ethylene glycol, 25% glycerol, and 0.05 M phosphate buffer) at 4°C until use. For western blotting and kit-based analyses, mice were decapitated, and the brains were isolated and stored at -80°C until use.

2.9 Immunohistochemistry, immunofluorescence, and NeuroTrace fluorescent analysis

For immunohistochemical analysis, brain sections were rinsed briefly in PBS and treated with 1% hydrogen peroxide for 15 min. The sections were incubated with rabbit anti-GFAP, Iba-1, iNOS, Cox-2, NeuN, and DCX (1:2000) overnight at 4°C in the presence of 0.3% Triton X-100 and normal goat serum (NGS). After rinsing in PBS, the sections were then incubated with biotinylated anti-rabbit IgG (1:200) for 90 min, rinsed, and incubated with ABC (1:100) for 1 h at room temperature. Heat-induced epitope retrieval was performed with sodium citrate buffer at a pH of 6.0 for CRH, and CRH receptor 1 (CRHR1). The sections were incubated with rabbit anti-SYN, CRH, and CRHR1 (1:1000) overnight at 4°C in the presence of 0.2% Triton X-100 and NGS. After rinsing in PBS, the sections were then incubated with biotinylated anti-rabbit IgG (1:500) for 90 min and with ABC (1:100) for 1 h at room temperature. Peroxidase activity was visualized by incubating sections with DAB in 0.05 M Tris-buffered saline (TBS, pH 7.6). After several rinses with PBS, sections were mounted on gelatin-coated slides, dehydrated, and coverslipped using histomount medium.

For immunofluorescence analysis, brain and skin sections were rinsed briefly in PBS, subjected to heat-induced epitope retrieval, and treated with 0.5% BSA for 30 min. The sections were incubated with rabbit anti-CRH, CRHR1, MT1, and melatonin (1:100-5000) overnight at 4°C in the presence of 0.3% Triton X-100 and NGS. They were then incubated for 2 h with an Alexa Fluor conjugated secondary antibody (diluted 1:500). Sections were

finally washed in PBS and mounted using Vectashield mounting medium containing DAPI.

For Fluoro-Jade® B labeling, which identifies degenerative changes in tissue, rehydrated sections were immediately transferred to a solution of 0.06% potassium permanganate for 10 min. The sections were then rinsed in distilled water for 2 min and placed in a 0.0004% Fluoro-Jade® B solution freshly diluted from a 0.01% stock solution of Fluoro-Jade® B with 0.1% acetic acid. After 20 min in the staining solution, the sections were rinsed through three changes of distilled water (1 min/change). Excess water was drained onto a paper towel, and the sections were then air dried on a slide warmer at 50° for at least 5 min. The dried sections were cleared by immersion in Histosol for at least 1 min, before coverslipping with Histomount. The sections were viewed with epifluorescence using blue (450–490 nm) excitation light as indicated by the manufacturer. Images were photographed at 40 × and 100 × magnification using an optical light microscope (Olympus Microscope System BX51; Olympus, Tokyo, Japan) equipped with a 20 × objective lens.

2.10 Measurement of MT1 receptor gene and protein expression levels

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies), and cDNA was synthesized using iScript cDNA synthesis kit following the manufacturer's instructions. Briefly, 20 µL of reaction volume containing 1 µg purified RNA, 5 µL 5x iScript reaction mix, 1 µL iScript reverse transcriptase, and the rest of nuclease free water was incubated for 5 min at 25°C, 20 min at 46°C, and 1 min at 95°C. The following oligonucleotides (5' to 3') were used as primers for RT-PCR: MT1 primers: GTC ATC AGC CTC GTC TGG CTC C (forward) and TCG TAC TTG CGG CTG TGG CAA ATG (reverse). The estimated product size of each reaction product of MT1 receptor were 248 bp. In addition, western blotting was performed according to previously published methods (Segrelles et al., 2002; Park et al., 2012; Park et al., 2014a; Park et al., 2014b; Park and Oh, 2015). The tissues were lysed by

homogenization with protease inhibitor cocktail (Roche, Mannheim, Germany) with added triple-detergent lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40) to detect MT1. The fractionation lysates were separated by 10–15% SDS-PAGE, and were then transferred to a membrane. The membranes were incubated with 5% skim milk in TBST for 1 h. Then they were incubated with primary antibody (1:1000 dilution) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody for 1 h. Immunoreactive bands were detected using an ECL detection kit, and a LAS-4000 mini system (Fujifilm Corporation, Tokyo, Japan) was used for visualization. The intensities of the bands were normalized to the β -actin band intensity using Multi Gauge software (Fujifilm Corporation, Tokyo, Japan). For the assessment of intra-experimental variability, three independent experiments were carried out in triplicate.

2.11 Measurement of LDH, CORT, UCN, POMC, CRH, CRHR, melatonin, IgE, MAPKs signaling, inflammatory cytokine array, IGFBP-3, MIP-2, LIX, and IL-1 α levels

LDH, CORT, UCN, POMC, CRH, CRHR, melatonin, IgE, MAP kinase (MAPK) signaling, IGFBP-3, macrophage inflammatory protein (MIP)-2, lipopolysaccharide-induced CXC chemokine (LIX), inflammatory cytokine array and interleukin-1 α (IL-1 α) were quantified using commercially available kits according to the instruction manuals, as was the inflammatory cytokine array.

2.12 Immortalized mouse hippocampal HT22 cell culture

The immortalized mouse hippocampal cell line (HT22) was kindly donated by Dr. Lim at KIOM in Korea. Cells were kept in DMEM supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin in an atmosphere of 95% air and 5% CO₂ at 37°C. All

experiments were conducted 12 h after the cells had been seeded on the 24-well plates at densities of 2×10^4 cells/well respectively. (See Fig. 1 for a timeline)

2.13 Rat primary hypothalamic neuronal culture

Clonetics® rat brain primary hypothalamic neuronal cells (RPHN) were purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA). Cells were kept in BulletKit™ supplemented with 2 mM L-glutamine, 50 µg/ml gentamicin and 37 ng/ml amphotericin, and 2% NSF-1 in an atmosphere of 95% air and 5% CO₂ at 37°C. The culture system was performed according to commercially available kits according to the instruction manuals. (See Fig. 1 for a timeline)

2.14 Measurement of cytotoxicity

HT22 and RPHN cells were treated simultaneously with CORT at concentrations of 10-100 µM for 24 h, and then release of LDH was determined using a CytoScan™ LDH-cytotoxicity assay kit, according to the instruction manual. The absorbance was measured using a spectrophotometer (Versamax microplate reader; Molecular Device, Sunnyvale, USA) at a wavelength of 570 nm, and was expressed as a percent of the value for the control.

2.15 Immunocytochemistry analysis

Cells were fixed through incubation with 4% paraformaldehyde (PFA) at room temperature for 30 min. Fixed cells were rinsed in PBS and then incubated overnight at 4°C with anti-pCREB and pERK antibody (dilution 1:500). They were then incubated with a biotinylated antibody IgG (1:1000 dilutions) for 60 min, followed by incubation in ABC solution for 1 h at room temperature or incubated for 2 h with an Alexa Fluor 594-conjugated goat anti-rabbit IgG (dilution 1:1000). Cells were finally washed in PBS and mounted using Vectashield

Mounting Medium containing DAPI. Representative images were taken using a fluorescence microscope (Olympus Microscope System BX51; Olympus, Tokyo, Japan). Data are presented as percentages of control values. For the assessment of intra-experimental variability, three independent experiments were carried out in triplicate.

2.16 Data analysis

We used the Search Tool for the Retrieval of Interacting Gene (STRING) software to construct a network model showing protein interactions based on known and predicted protein-protein interactions. In addition, protein ontology and functional relationships were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

2.17 Statistical analysis

All statistical parameters were calculated using Graphpad Prism 5.0 software (Graphpad Software, San Diego, CA, USA). Values are expressed as means $\pm$ standard error of the mean (S.E.M.). Statistical comparisons between the different treatments were performed using one-way ANOVA with Tukey's multiple comparison post test. p values of < 0.05 were considered to be statistically significant.

3. Results

3.1 Effects of DNCB on skin tissue integrity

We first assessed the clinical score, itching behavior, IgE, hematoxylin/eosin-stained skin sections, and skin LDH levels to determine if DNCB itself caused cutaneous damage. Briefly, the 2% DNCB group demonstrated increased clinical score (Fig. 2A; 2.6 ± 0.5 to 4.4 ± 0.4 score), itching behavior (Fig. 2B; 17.2 ± 2.9 min), IgE levels (Fig. 2C; 165.08 ± 20.17 to 260.77 ± 10.17 ng/mL), epidermal thickness (Fig. 2D), as well as LDH levels (Fig. 2E; 287.75 ± 72.84 to $568.35 \pm 47.29\%$ of the corresponding control) indicative of skin damage. In addition, DNCB-induced skin damage was reversed in the DNCB + dexamethasone group (Fig. 2A, C, and Supplementary Fig. 2).

3.2 Effects of DNCB on HPA axis activity biomarkers in skin, plasma, and brain samples

Second, we measured CRH, CRHR1, UCN, POMC, ACTH, and CORT in skin to assess peripheral HPA axis activity. Qualitatively, we found CRH expression in epidermal and mast cells, and CRHR1 in the epidermal and subcutis layers. CRH and CRHR1 were significantly increased by 2% DNCB (Fig. 2D and F; CRH, $835.03 \pm 95.22\%$ of the corresponding control; CRHR1, $504.94 \pm 37.53\%$ of the corresponding control). The 0.5% to 2% DNCB groups demonstrated significantly increased UCN (Fig. 3A; 141.86 ± 50.33 to $360.61 \pm 55.68\%$ of the corresponding control), POMC (Fig. 3B; 183.11 ± 43.47 to $520.32 \pm 91.30\%$ of the corresponding control), ACTH (Fig. 3C; 112.94 ± 11.61 to $291.91 \pm 42.81\%$ of the corresponding control), and CORT (Fig. 3D; 5.59 ± 0.03 to 11.65 ± 0.28 ng/mL) in skin.

We further assessed HPA axis activity by measuring UCN, POMC, ACTH, and CORT levels in plasma. As seen in fig. 2, exposure to 0.5 to 2% DNCB significantly increased both UCN (Fig. 3E; 85.33 ± 2.85 to $150.29 \pm 15.29\%$ of the corresponding control),

POMC (Fig. 3F; 91.57 ± 8.19 to $179.25 \pm 8.56\%$ of the corresponding control), ACTH (Fig. 3G; 115.69 ± 26.01 to $192.17 \pm 12.32\%$ of the corresponding control), and CORT (Fig. 3H; 306.81 ± 41.36 to 402.71 ± 73.97 ng/mL).

In the brain, we measured CRH, CRHR1, UCN, POMC, ACTH, and CORT levels in samples of the hypothalamus and hippocampus. The 0.5% to 2% DNCB group had increased CRH and CRHR1 levels in the hippocampus (Fig. 4A, B, and C; $118.51 \pm 2.40\%$ and $123.33 \pm 5.53\%$ of the corresponding control, respectively) and hypothalamus (Fig. 4A, D, and E; by $121.86 \pm 2.29\%$ and $206.68 \pm 13.16\%$ of the corresponding control, respectively). Additionally, the 0.5% to 2% DNCB groups demonstrated significantly increased UCN (Fig. 5A; 85.33 ± 2.85 to $150.29 \pm 15.29\%$ of the corresponding control), POMC (Fig. 5B; 91.57 ± 8.19 to $179.25 \pm 8.56\%$ of the corresponding control), ACTH (Fig. 5C: 115.69 ± 26.01 to $192.17 \pm 12.32\%$ of the corresponding control), and CORT (Fig. 5D; 348.06 ± 4.12 to 475.76 ± 16.46 ng/mL) in brain. However, the group showed no significant increases CORT levels in the cortex (Supplementary Fig. 3D).

3.3 Effects of DNCB on stress response pERK-pCREB in the hippocampus

We assessed ERK-CREB pathway activity in the hippocampus. Exposure of skin to 0.5% to 2% DNCB significantly increased pERK (Fig. 6A; 157.62 ± 11.41 to $249.85 \pm 23.61\%$ of the corresponding control) and pCREB (Fig. 6B and C; $227.56 \pm 32.36\%$ of the corresponding control) in the hippocampus (Fig. 6B and C). Moreover, we confirmed the pERK and pCREB levels in a mouse hippocampal HT22 cell line. Cell viability of HT22 cells was not affected by CORT treatment at 10 to 50 μ M, according to an LDH assay 24 h later, but was reduced by 100 μ M CORT exposure (Fig. 7A; 103.08 ± 6.14 to $166.32 \pm 5.82\%$ of the corresponding control). As seen in fig. 7A, C, and D, exposure to CORT significantly increased pERK (Fig. 7A and C; 106.89 ± 1.21 to $147.60 \pm 4.24\%$ of the corresponding control) and pCREB (Fig.

7A and D; 423.95 ± 53.91 to $706.29 \pm 25.02\%$ of the corresponding control) levels.

3.4 Effects of DNCB on melatonin in the hypothalamus

Most of the mice slept during the daytime and were active at night. Before this study, we checked typical melatonin levels using hypothalamus of NC/Nga mice brain. Because rodents are nocturnal. On P.M 15:00 to 16:00, the melatonin levels is 461.48 pg/mL in hypothalamus of brain of NC/Nga mice. Moreover, on A.M 02:00 to 03:00, melatonin levels is 49.12 pg/mL in hypothalamus of brain of NC/Nga mice. This case is did not consistent with a previous report that showed that pineal melatonin content in CBA mice decreased to basal levels after 5 min exposure to 100Lx light (this system mean the morning)⁴². We guess that mice didn't active it in the morning. We measured MT1 receptor (MT1R) and melatonin levels using immunohistochemistry, PCR, and western blotting. As seen in fig. 8, exposure to 2% DNCB significantly decreased both MT1R (RNA, Fig. 8B, $45.61 \pm 1.04\%$ of the corresponding controls; Protein levels, Fig. 8A and C, 90.09 ± 2.42 to $52.71 \pm 8.95\%$ of the corresponding controls) in the hypothalamus. However, the 2% DNCB group showed only weak decreases in the hippocampus (Fig. 8B). The 0.5% to 2% DNCB groups demonstrated significantly decreased melatonin in skin (Fig. 8D; 9.89 ± 1.43 to 3.53 ± 0.65 pg/mL), plasma (Fig. 8E; 13.92 ± 0.66 to 6.39 ± 0.69 pg/mL), hippocampus (Fig. 8F; 34.75 ± 5.51 to 12.79 ± 2.42 pg/mL) and hypothalamus (Fig. 8F; 44.04 ± 6.16 to 20.21 ± 3.71 pg/mL) samples. We confirmed the MT1R and melatonin levels in rat primary hypothalamic neuron cultures. The 10-20 μ M CORT groups demonstrated significantly decreased melatonin (Fig. 8G; 1.82 ± 2.94 to 1.15 ± 1.28 pg/mL) levels. We also assessed melatonin in the plasma and brain of NC/Nga mice (the CORT AD-like model). As seen in fig. 8H, exposure to 20 mg/kg CORT significantly increased melatonin in plasma (5.58 ± 1.38 pg/mL) and in the hypothalamus (27.02 ± 5.27 pg/mL).

3.5 Effects of melatonin on DNCB-induced skin tissue integrity

To investigate the effect of melatonin on DNCB-induced AD model skin, we assessed the clinical score, itching behavior, IgE, and hematoxylin/eosin staining in skin sections. Treatment with DNCB significantly increased clinical scores (Fig. 9B; 4.7 ± 0.2 score), itching behavior (Fig. 9C; 44.0 ± 7.8 min), IgE (Fig. 9D; 324.15 ± 19.34 ng/mL), and epidermal thickness (Fig. 9A) relative to the control mice, while treatment with 20 mg/kg melatonin reduced the DNCB-induced clinical skin severity scores (Fig. 9B; 2.5 ± 0.3 score) and itching behavior (Fig. 9C; 18.4 ± 3.2 min). In addition, treatment with 20 mg/kg melatonin reduced the DNCB-induced IgE levels (Fig. 9D; 130.56 ± 17.45 ng/mL) and epidermal thickness (Fig. 9A).

3.6 Effects of melatonin on DNCB-induced HPA axis activity biomarkers

To determine whether melatonin affected AD-related stress responses, we determined CRH, CRHR, and CORT levels in skin, plasma, and brain. Treatment with DNCB significantly increased the levels of CRH, CRHR1, and CORT (Fig. 9F; in skin; 20.80 ± 2.55 ng/mL, Fig. 9G; in plasma; 583.47 ± 56.69 ng/mL, and Fig. 9H; in the hippocampus; 367.94 ± 6.67 ng/mL and in the hypothalamus; 384.85 ± 23.68 ng/mL) levels compared with the control group, while treatment with 20 mg/kg melatonin reduced DNCB-induced CRH, CRHR1, and CORT (Fig. 9F; in skin; 12.68 ± 1.41 ng/mL, Fig. 9G; in plasma; 350.02 ± 18.47 ng/mL, and Fig. 9H; in the hippocampus; 287.39 ± 23.29 ng/mL and Fig. 9H; in the hypothalamus; 310.94 ± 7.40 ng/mL). Additionally, we checked MT1R levels immunohistochemically, and found that exposure to 2% DNCB significantly decreased MT1R relative to the corresponding controls, while addition of melatonin increased MT1R, in the hypothalamus (fig. 9E).

3.7 Effects of melatonin on DNCB-induced glial activation and expression of iNOS and Cox-2 levels in the hippocampus and hypothalamus

To investigate the effects of melatonin against DNCB-induced glial responses, we measured activation of astrocytes (via GFAP) and microglia (via Iba-1). Treatment with DNCB significantly increased GFAP (Fig. 10A and B; $186.64 \pm 7.82\%$ of the corresponding control) and Iba-1 expression (Fig. 10A and C; $451.85 \pm 62.42\%$ of the corresponding control), while treatment with melatonin reduced the DNCB-induced GFAP (Fig. 10A and B; $115.41 \pm 16.58\%$ of the corresponding control) and Iba-1 expression (Fig. 10A and C; $187.32 \pm 11.56\%$ of the corresponding control) in the hippocampus. In addition, to determine the effects of melatonin against DNCB-induced neuroinflammation, we measured Cox-2 and iNOS levels. Treatment with DNCB significantly increased Cox-2 (Fig. 10D and E; $116.43 \pm 2.42\%$ of the corresponding control) and iNOS (Fig. 10D and E; $124.61 \pm 5.46\%$ of the corresponding control), while treatment with melatonin reduced both DNCB-induced Cox-2 (Fig. 10D and E; $105.44 \pm 3.12\%$ of the corresponding control) and iNOS (Fig. 10D and F; $107.36 \pm 4.86\%$ of the corresponding control) in the hippocampus. Treatment with DNCB significantly increased GFAP (Fig. 10G and H; $489.73 \pm 37.46\%$ of the corresponding control) and Iba-1 (Fig. 10G and I; $403.95 \pm 58.03\%$ of the corresponding control) expression, while treatment with melatonin reduced DNCB-induced GFAP (Fig. 10G and H; 307.57 ± 41.10) and Iba-1 (Fig. 10G and I; $202.05 \pm 11.83\%$ of the corresponding control) expression in the hypothalamus. Treatment with DNCB significantly increased hypothalamic Cox-2 (Fig. 10J and K; $165.41 \pm 15.92\%$ of the corresponding control) and iNOS (Fig. 10J and L; $140.69 \pm 5.18\%$ of the corresponding control) expression, while treatment with melatonin reduced the hypothalamic DNCB-induced Cox-2 (Fig. 10J and K; $104.87 \pm 3.33\%$ of the corresponding control) and iNOS (Fig. 10J and L; $107.70 \pm 2.59\%$ of the corresponding control) expression.

3.8 Effects of melatonin on DNCB-induced hippocampal markers of neuronal degeneration and development

To investigate the effects of melatonin against DNCB-induced neuronal damage in the hippocampus, we analyzed NeuN and Fluoro-Jade® B labeling. DNCB significantly decreased NeuN intensity (Fig. 11A and B; DG; $92.49 \pm 0.27\%$ of the corresponding control and CA3; $92.21 \pm 0.20\%$ of the corresponding control) and numbers of NeuN-positive nuclei (Fig. 10A and B; DG; $73.25 \pm 15.28\%$ of the corresponding control and CA3; $67.63 \pm 7.59\%$ of the corresponding control). Fluoro-Jade® B, which labels dying cells and dendrites, was significantly more intense (Fig. 10C and D; DG; $573.24 \pm 61.67\%$ of the corresponding control and CA3; $785.52 \pm 92.51\%$ of the corresponding control). Melatonin significantly reversed the DNCB-induced weakening of NeuN intensity (Fig. 11A and B; DG; $97.83 \pm 2.09\%$ of the corresponding control and CA3; $98.56 \pm 1.84\%$ of the corresponding control) and reduced NeuN counts (Fig. 10A and B; DG; $84.54 \pm 3.03\%$ of the corresponding control and CA3; $89.78 \pm 9.87\%$ of the corresponding control); melatonin also partially reduced the DNCB-related increase in Fluoro-Jade® B labeling intensity (Fig. 10C and D; DG; $344.42 \pm 28.30\%$ of the corresponding control and CA3; $346.94 \pm 62.74\%$ of the corresponding control). DCX labeling and dendrite length, markers of neuronal development, were decreased in the 2% DNCB group (Fig. 10E and F; count, $76.82 \pm 5.71\%$ of the corresponding control; dendrite length, $51.09 \pm 5.43\%$ of the corresponding control); treatment with melatonin reversed these changes (Fig. 10E and F; count, $94.53 \pm 1.98\%$ of the corresponding control; dendrite length, $86.65 \pm 5.01\%$ of the corresponding control).

3.9 Effects of DNCB on synaptic activity in the hippocampal CA3 area

We analyzed synaptic activity in the hippocampus related to DNCB exposure by quantifying

SYN. Exposure of skin to 2% DNCB had no significant effect on the DG, but SYN labeling was significantly lower than that in controls ($85.10 \pm 4.22\%$, $p < 0.05$) in the CA3 region of the hippocampus (Fig. 10G and H). However, melatonin treatment group slightly inhibited against this effect ($91.35 \pm 8.62\%$).

3.10 Effect of melatonin on DNCB-induced memory impairment quantified via the modified Y-maze

Because the scratching behavior interfered with the rodent cognitive tasks, we attempted to use the modified Y-maze in this study to assess cognition. The modified Y-maze is quantified according to the number of arm entries, but does not limit the latency time (40 total entries were used per animal) (Fig. 12). Spontaneous alternations were significantly less frequent in the DNCB group ($34.21 \pm 2.76\%$) than those in the control group ($45.26 \pm 0.98\%$). Melatonin treatment restored spontaneous alternations to approximately control levels ($42.11 \pm 2.04\%$). An analysis of the latency time determined that DNCB significantly increased activity ($3638.00 \pm 310.39\%$ of the corresponding control), but that there were no differences between the DNCB and the melatonin-treated groups ($2936.80 \pm 253.33\%$).

3.11 Effects of melatonin on DNCB-induced inflammatory cytokines in the brain

To investigate the mechanisms by which DNCB induces neuroinflammation, the profiles of various inflammatory cytokine-related proteins were examined in the hippocampus, midbrain, cortex, and striatum (Fig. 13). The array results, as seen in figure 9, demonstrated significant increases in the expression of IGFBP-3, macrophage colony-stimulating factor (M-CSF), MIP-2, MIP-3 β , MIP-3 α , PF-4, and TNF-alpha (1.3 fold or more; red zone) in the hippocampus of mice in the 2% DNCB group. Also in the DNCB group, expression was marginally increased for CXCL16, IL-1 α , IL12p70, L-selectin, Ltn, TARC, and TPO (1.2

fold or more; orange zone). Interestingly, as seen in fig. 13A and B, exposure to 2% DNCB significantly increased cytokine responses only in the hippocampus and cortex. We analyzed protein-protein interactions using the STRING database, and potential molecular mechanisms using the KEGG database (Fig. 13C). We found functional relationships among the proteins primarily related to neuronal damage/repair mechanisms, including cytokine/cytokine receptor interactions, chemokine signaling, and IL-17 signaling (Supplementary Fig. 5). To confirm these findings, we tested the effects of melatonin on several types of DNCB-induced cytokine expression. Treatment with DNCB significantly increased IGFBP-3 (Fig. 13E; $558.98 \pm 79.85\%$ of the corresponding control), MIP-2 (Fig. 13F; $155.78 \pm 3.42\%$ of the corresponding control), IL-1alpha (Fig. 13G; $597.15 \pm 27.77\%$ of the corresponding control), LIX (Fig. 13H; $148.21 \pm 21.24\%$ of the corresponding control), and TNF-alpha (Fig. 13I; $197.92 \pm 21.12\%$ of the corresponding control), while treatment with melatonin decreased DNCB-induced increases in IGFBP-3 ($281.11 \pm 7.60\%$ of the corresponding control), MIP-2 ($107.57 \pm 2.37\%$ of the corresponding control), IL-1alpha ($261.31 \pm 2.84\%$ of the corresponding control), LIX ($108.40 \pm 6.32\%$ of the corresponding control), and TNF-alpha ($134.62 \pm 16.28\%$ of the corresponding control) in the hippocampus.

3.12 Effects of melatonin on CORT-induced skin tissue integrity

To assess the effects of melatonin on CORT-induced melatonin levels, we quantified melatonin in skin, plasma, and both the hippocampus and hypothalamus (see “Effects of DNCB on melatonin in the hypothalamus” above) tissue. Treatment with CORT significantly decreased melatonin levels in skin (Fig. 14A; 3.75 ± 0.94 pg/mL), plasma (Fig. 14B; 5.18 ± 0.68 pg/mL), hippocampus (Fig. 14C; 16.90 ± 2.23 pg/mL), and hypothalamus (Fig. 14C; 31.36 ± 7.79 pg/mL). Next, to quantify the effect of melatonin, we analyzed LDH levels, IgE levels, and hematoxylin/eosin staining of epidermal thickening, as well as IL-4, IL-13, and

TNF- α , in CORT-induced, AD-like skin sections. CORT significantly increased LDH level (Fig. 14E; $198.88 \pm 6.71\%$ of the corresponding control), IgE level (Fig. 14F; 135.64 ± 22.25 ng/mL), epidermal thickness (Fig. 14D), IL-4 level (Fig. 14D and G; $214.24 \pm 25.69\%$ of the corresponding control), IL-13 level (Fig. 14D and G; $249.06 \pm 21.21\%$ of the corresponding control), and TNF- α level (Fig. 14D and G; $396.24 \pm 44.41\%$ of the corresponding control), while treatment with 20 mg/kg melatonin reduced the CORT-induced levels of LDH ($137.06 \pm 13.11\%$ of the corresponding control), IgE level (75.80 ± 8.74 ng/mL), epidermal thickness, IL-4 level ($93.37 \pm 20.83\%$ of the corresponding control), IL-13 level ($138.11 \pm 16.80\%$ of the corresponding control), and TNF- α level ($225.16 \pm 26.63\%$ of the corresponding control).

4. Discussion

Our data demonstrated that atopy-induced stress significantly accelerated neuronal damage and increased neuroinflammation, primarily via exacerbating a chronic state of high glucocorticoid activity and suppressing melatonin-mediated feedback. Atopic stress (1) induced CRH-mediated signaling and suppressed melatonin signaling in skin, plasma, and brain; (2) increased astrocyte and microglial activation and expression of proinflammatory molecules; and (3) increased expression of indicators of neuronal loss. Melatonin reversed the above atopy symptoms and neuronal damage through a glucocorticoid-related mechanism, given that the responses were confirmed in a CORT-induced AD mouse model. Our findings suggest that atopic stress might contribute to neuronal dysfunction by causing or enhancing neuroinflammation and neurodegeneration, primarily via dysfunction of glucocorticoid/melatonin signaling pathways.

Stressors exert their effects on skin mainly through the HPA axis and CORT production^{22, 43-44}. We found that DNCB increased expression of several of the molecules in this pathway, which reflects the similar results of a previous study. Notably, Shin et al. found

that continuous high CORT levels related to psychological stress can trigger AD-like skin lesions^{35, 45}. However, pretreatment with CRH inhibited scratching behavior in a water avoidance stress mouse model³⁵. We found CRH expression in epidermal and mast cells, and CRHR1 expression in the epidermal and subcutis layers (Fig. 2). Previous studies have demonstrated a similar distribution under high-stress conditions; one such study showed that CRH was produced by stressed epidermal, follicular and mast cells under physiological stress⁴⁶⁻⁴⁹. CRHR1 was found to be expressed in all major cellular populations of the epidermis, dermis, and subcutis in humans. Furthermore, human melanocytes and dermal fibroblasts respond to CRH signaling via cAMP-mediated signal transduction to induce ACTH and CORT production^{46, 50}.

This study demonstrated that HPA axis activity in the hypothalamus and hippocampus were sensitive to atopic stress. Both regions are important sites of action for corticosteroid-mediated stress responses⁵¹⁻⁵². In particular, the hippocampus is vulnerable to chronic corticosteroid-induced damage^{51, 53}. In addition, chronic stress modeled in animals, or related to conditions such as post-traumatic stress disorder, will cause hippocampal atrophy and volume reduction are seen in both post-traumatic stress disorder and in animal models of chronic stress⁵³⁻⁵⁴. We found significantly higher expression of CRH, CRHR1, UCN, POMC, ACTH, and CORT in the hypothalamus and hippocampus (Fig. 4 and 5), all of which were reversed when dexamethasone was applied with 2% DNCB (supplementary fig. 2). Dexamethasone acutely and dose-dependently depletes endogenous glucocorticoids, and downregulates glucocorticoid production in the brain with repeated injection⁵⁵. However, according to Sousa et al., dexamethasone acting on corticosteroid receptor II probably causes neuronal loss in the hippocampal formation in rats⁵⁶. Thus, we think to need the further detail study. Hypothalamic CRH neurons are regulated in part by the hippocampus as part of the negative feedback loop regulating plasma corticosteroid levels⁵⁵. Corticosteroid-induced

hippocampal damage impairs the negative feedback loop, and potentially increases high corticosteroid levels even further⁵⁷⁻⁵⁸.

Corticosteroid-mediated psychological stress responses utilize various signal transduction systems, including the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (Erk1/2)⁵⁹. Studies have shown Erk1/2 to respond to certain stressful stimuli, such as ischemia, visceral pain, and electroconvulsive shock⁶⁰. ERK activation is associated with the transcriptional factor CREB in hippocampal neurons or slices, in which it activates c-fos via cyclic-AMP response element (CRE) sites in the promoter regions⁶⁰. Stress has recently been reported to enhance CREB phosphorylation (pCREB, the active form of CREB)⁶⁰⁻⁶¹. In this study, we found significant increases in pERK/pCREB in the DG and CA3 regions of the hippocampus similar to those seen in the psychological stress response (Fig. 6). In hippocampal HT22 cell cultures, CORT stimulation significantly increased ERK/CREB expression (Fig. 7).

Melatonin also interacts closely with glucocorticoid systems, and both have immunoregulatory effects. In fact, direct communication between the pineal and adrenal glands has been well-established. Melatonin is important for immune function under both physiological and pathophysiological conditions⁶²⁻⁶³. For example, the higher melatonin at night increases the capacity of macrophages to phagocytose antigens in mice⁶⁴. The relationship between melatonin, cortisol, and the sleep cycle is delineated by the finding that the nightly circadian rise in melatonin correlates with the nightly circadian drop in cortisol⁶⁵⁻⁶⁶. Any chronic, late-night stressor can result in excessively high night-time cortisol; shift-work is an example of such a stressor⁶⁷. This chronic excess of cortisol at night may impair the normal circadian corticosteroid increase in the morning⁶⁷. Indeed, Kim et al. identified a dramatic decrease in cerebrospinal fluid melatonin levels in both elderly control patients and those with Alzheimer's disease⁶⁸. Supplementation of melatonin may improve sleep and

behavioral disturbances in patients with neurodegenerative disease; this may also be true for patients with atopic dermatitis, given the indications of sleep disturbances in such patients and their positive sleep and skin lesion responses to increased melatonin secretion^{30, 68}. Our results showed significantly decreased MT1R and melatonin expression in the hypothalamus (Fig. 8), as well as significantly decreased melatonin expression in rat primary hypothalamic neurons and NC/Nga mice exposed to CORT (Fig. 8). We also found that subchronic melatonin reduced atopy scores and reversed CRH, CRHR1, and CORT disturbances in DNCB-treated mice (Fig. 9). Taken together, atopic stress enhanced the disruption of normal glucocorticoid/melatonin regulation.

Melatonin also mediates glial neuroimmune responses. Recent work has shown that 6 days of activity stress (high activity combined with restricted food access) in rats caused a 30% increase in GFAP-immunoreactive astrocytes within the hippocampus compared to unstressed controls¹⁶. Long term CORT injection (daily for 4 weeks) also increased the number of astrocytes and somal volume in the hippocampus of mouse brain¹⁶. We found significant increases in activated glia in the hippocampus and hypothalamus of the DNCB-induced AD model mice (Fig. 10). The present findings suggest that the hippocampal glial response to chronic stress may be similar to the response found in pathological hippocampal environments, such as in ischemic conditions. We also found increased microglial activity in the AD model mice, as well as increased inflammation-related enzyme expression. These data are indicative of the innate immune response in the brain, in which microglia are the “front line” defense (Fig. 10). Not surprisingly, the same microglial response occurs with chronic stress and high glucocorticoids, supporting our hypothesis of an atopy-chronic stress positive feedback loop³⁰.

Our investigation of inflammatory cytokine expression in atopic stress identified several upregulated cytokines, including IGFBP-3, LIX, M-CSF, MIP-2, MIP-3 β , MIP-3 α ,

PF-4, and TNF- α (Fig. 13)⁶⁹⁻⁷⁰. While all eight mediate neuroinflammation, based on our analysis of their interactions and KEGG-identified mechanisms, IGFBP-3 is particularly noteworthy in that it usually appears to act as an anti-inflammatory, NF- κ B pathway inhibitor⁷¹. However, recent studies demonstrated increased IGFBP-3 in astrocytes, hyperphosphorylated tau-positive neurons, and around some β -amyloid plaques in patients with Alzheimer's disease, suggesting that it might conversely play a pro-inflammatory role in Alzheimer's disease-related neuroinflammation⁷²⁻⁷⁴.

We found slight decreases in NeuN and Nissl staining suggestive of mild neuronal loss in the CA3 region, and increased Fluoro-Jade B labeling indicative of neurodegeneration, and reduced DCX labeling indicative of reduced migration (Fig. 11 and supplementary fig. 4), all of which are symptoms of inflammatory damage. Consistent with our results, Kim et al. demonstrated that exogenous glucocorticoids can adversely affect both hippocampal morphology and function during cognitive tasks in humans⁷⁵⁻⁷⁷. Moreover, high glucocorticoid levels produce atrophic changes in the hippocampus similar to those exhibited during aging; in fact, the hippocampus may become even more vulnerable to glucocorticoid damage with increased age, suggesting that glucocorticoids effectively accelerate the aging process in the brain⁷⁵.

To assess functional impairment, we analyzed SYN, markers of synaptic structural plasticity that are critical to proper hippocampal function⁷⁸⁻⁷⁹. We found significant decreases in both markers in the CA3 region of the hippocampus (Fig. 11). This correlates with previous work demonstrating that SYN expression decreased in the CA3 region of the hippocampus after long-term stress or CORT injection (Fig. 11). Decreased SYN expression was in turn correlated with neuronal atrophy, which may be the underlying mechanism of stress or CORT-induced hippocampal dysfunction⁷⁹. Our results demonstrate a hypothetical pathway through which HPA hyperactivity related to atopic stress may induce

neuroinflammation and the corresponding hippocampal damage it causes, or it could accelerate the effects of neuroinflammation produced by another disorder, such as Alzheimer's disease.

Several lines of evidence support this accelerated aging effect. As stated, melatonin secretion is both decreased with age and inhibited by glucocorticoids. DNCB-induced NC/Nga mice treated with melatonin showed dramatically inhibited atopy symptoms (clinical skin severity scores, epidermal thickness, and IgE levels), as well as psychological stress-related protein expression and CORT release (Fig. 9). Moreover, melatonin also inhibited neuroimmune activation (glial activity), neuroinflammatory reactions, neurodegeneration, and hippocampal damage (Fig. 10 and 11). Moreover, in this pilot study, we found that melatonin treatment in DNCB-induced NC/Nga mice inhibited memory impairments identified by the modified Y-maze test (Fig. 12). In addition to the DNCB-induced AD animal model, we developed a CORT-based AD animal model to verify the results of the DNCB model. The AD model mice with injected CORT showed dramatically accelerated atopy symptoms and related cytokine (IL-4, IL-13, and TNF- α) levels, supporting the likelihood of the excess glucocorticoid mechanism and its inhibition by melatonin (Fig. 14). Our results demonstrate a hypothetical pathway through which CORT/melatonin dysfunction related to atopic stress may induce neuroinflammation and its correlated hippocampal damage, or accelerate the effects of neuroinflammation produced by another disorder, such as neurodegenerative disease or advanced age.

This study had several limitations. First, we did not assess whether atopy-related stress induced cognitive impairment or other behavioral markers of chronic stress; the itching behavior interfered with the rodent cognitive tasks. We attempted to solve this issue with the modified Y-maze behavioral test, which does not control the latency time (only the number of entries). Second, we could not clearly separate the effects of psychological versus

physiological stress. We attempted to solve this issue with the CORT-induced atopy response model, which does not have the skin damage-stress. However, future studies will be needed to analyze the specific contributions of each type of stress, as well as their potential cognitive and behavioral impairments.

In summary, we demonstrated that atopy-induced stress significantly accelerated dysfunction of the HPA axis, increased neuroinflammation, and increased neuronal damage (Supplementary fig. 1). Atopic stress induced CRH, UCN, POMC, ACTH, CORT, and ERK/CREB response signaling, which reduced neuroendocrine melatonin signals. Moreover, its conditions accelerated glia and microglia activation, neuroinflammatory response signaling, and increased expression of indicators of neuronal loss. In DNCB-induced and CORT-induced AD of NC/Nga mice models, we further observed that melatonin inhibited atopy symptom and reduced neurotoxicity by inhibiting neuroinflammatory response via regulation of HPA axis signaling. Thus, we suggest that CORT/melatonin disequilibrium might contribute to neuronal dysfunction by causing or enhancing neurodegeneration.

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Author contributions

Dr. Park conceived the idea and designed the experiments. Dr. Park and Dr. Lee performed the experiments. Dr. Kim contributed ICR mice and ELISA kits and provided helpful recommendations. Dr. Park and Dr. Oh performed data analysis. Dr. Park, Dr. Lee, Dr. Oh,

and Dr. Kim assisted in data interpretation and manuscript preparation. Dr. Park wrote the entire manuscript. All authors commented on and approved the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

Figure 1. Summary of the experimental design.

Figure 2. Effects of DNCB on skin integrity. The levels of clinical skin severity scores (A) and itching behavior (B) were observed; IgE (C) and LDH (E) were quantified by ELISA kits. Representative hematoxylin and eosin staining photomicrographs are shown (D). Effects of DNCB on expression of CRH in skin. The levels of CRH (D, F) and CRHR1 (D, F) were quantified by immunofluorescence. Values are means $\pm$ standard error of the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control group.

Figure 3. Effects of DNCB on CRH-related HPA axis activities in skin and plasma. The levels of UCN (A), POMC (B), ACTH (C), and CORT (D) in skin and UCN (E), POMC (F), ACTH (G), and CORT (H) were quantified by ELISA kit. Values are means $\pm$ standard error of the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control group.

Figure 4. Effects of DNCB on expression of the CRH signaling in the hippocampus and hypothalamus. The levels of CRH (B, D) and CRHR (C, E) were measured by immunofluorescence. Representative photomicrographs are shown (A). Values are means $\pm$ standard error of the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control group.

Figure 5. Effects of DNCB on expression of the CRH-related HPA axis cascade in the hippocampus and hypothalamus. The levels of UCN (A), POMC (B), ACTH (C), and CORT (D) were measured by ELISA kit. Values are means $\pm$ standard error of the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control group.

Figure 6. Effects of DNCB on expression of the stress response pERK-pCREB signaling in the hippocampus. The expression of pERK (A) and pCreB (C) were measured by ELISA kit or immunofluorescence. Representative photomicrographs are shown (B). Values are means $\pm$ standard error of the mean. * $P < 0.05$ and *** $P < 0.001$ compared with the control group.

Figure 7. Effects of CORT on expression of signaling in an immortalized mouse hippocampal HT22 cell line. The levels of cell toxicity (B), pERK (C), and pCreB (D) were quantified by immunofluorescence or ELISA kit. Representative photomicrographs are shown (A). Values are means $\pm$ standard error of the mean. ** $P < 0.01$ and *** $P < 0.001$ compared with the control group.

Figure 8. Effects of DNCB on expression of the MT1 receptor and melatonin contents in the hypothalamus. The expression of MT1R RNA (B) and MT1R protein (C) levels were measured by PCR and western blotting analysis. Representative photomicrographs are shown (A). In addition, the melatonin levels in skin (D), plasma (E), and brain (F) were measured by ELISA kit. Moreover, to confirm the melatonin content in CORT-induced models, we analyzed levels in Clonetics® rat brain primary hypothalamic neuronal cells (G) and NC/Nga mice (H).

Figure 9. Effects of melatonin on DNCB-induced skin integrity. The levels of hematoxylin and eosin staining (A), clinical skin severity scores (B), scratching behavior (C), and IgE (D) were quantified by staining or ELISA kit. Effects of melatonin on DNCB-induced HPA axis activities in skin or plasma. The levels of CRH, CRHR1 (E), and skin CORT (F), plasma CORT (G), brain CORT (H) were quantified by immunofluorescence or ELISA kit. Moreover, the levels of MT1 receptor were measured by immunofluorescence. Values are means $\pm$ standard error of the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with the 2% DNCB-alone group.

Figure 10. Effects of melatonin on DNCB-induced glial activation and neuroinflammation in the hippocampus and hypothalamus. The activation of astrocytes and microglia in the hippocampus (A, B, C) and hypothalamus (G, H, I) were measured by GFAP and Iba-1 immunohistochemistry. Levels of Cox-2 and iNOS in hippocampus (D, E, F) and hypothalamus (J, K, L) were measured by immunohistochemistry. Values are means $\pm$ standard error of the mean. ** $P < 0.01$ and *** $P < 0.001$ compared with the control group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with the 2% DNCB-alone group.

Figure 11. Effects of melatonin on DNCB-induced neurodegeneration and pre-synapse in the hippocampus. The neuronal damage of NeuN (A, B) in the hippocampus were measured by NeuN immunohistochemistry. Degenerating neurons and neuronal migration were measured by Fluoro-Jade B stain (C, D) and DCX (E, F) immunohistochemistry. (G, H) The synaptic activity measured by SYN immunohistochemistry in the CA3 region of hippocampus. Values are means $\pm$ standard error of the mean. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the control group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with the 2% DNCB-alone group.

Figure 12. Effect of melatonin on DNCB-induced memory impairment. Modified Y-maze tests were performed on day 55 after DNCB exposure. The spontaneous alternation index (A) and latency time (B) and were recorded. Values are means $\pm$ standard error of the mean. **P<0.01 and ***P<0.001 compared with the control group; #P<0.05 compared with the 2% DNCB-alone group.

Figure 13. Protein array data quantifying inflammatory cytokines the hippocampus, midbrain, cerebral cortex, and striatum. Representative images of the array results from the 2% DNCB group; the grid details the proteins represented by each dot in the array (A). Densitometric ratios of the arrays showed differences in the inflammatory cytokine markers (B). Moreover, a network of protein-protein interactions and functional relationships generated from proteins using STRING software and the KEGG database (C). IGFBP-3 measured in the hippocampus using immunohistochemistry (D). Effects of melatonin on the DNCB-induced cytokines IGFBP-3 (E), MIP-2 (F), IL- α (G), LIX (H), and TNF- α (I) in the hippocampus. Values are means $\pm$ standard error of the mean. *P<0.05 and ***P<0.001 compared with the control group; ##P<0.01 and ###P<0.001 compared with the 2% DNCB-alone group.

Figure 14. Effects of melatonin on CORT-induced skin integrity. The levels of melatonin in skin, plasma, and regional brain samples (A, B, and C), LDH (D), and IgE (E) were quantified by ELISA kits. Representative hematoxylin and eosin staining to demonstrate epidermal thickness, IL-4, IL-13, and TNF- α photomicrographs are shown (F). Effects of melatonin on CORT-induced IL-4, IL-13, and TNF- α levels measured by immunofluorescence or ELISA kits (G). Values are means $\pm$ standard error of the mean. *P<0.05, **P<0.01, and ***P<0.001 compared with the control group; ##P<0.01 compared

with the CORT-alone group.

Supplementary figure 1. Proposed mechanism of action for melatonin-related inhibition of brain damage induced by atopic psychological stress. DNCB, 2,4-dinitrochlorobenzene; CRH, corticotropin-releasing hormone; UCN, Urocortin; CRHR1, corticotropin-releasing hormone receptor 1; POMC, proopiomelanocortin; ACTH, adrenocorticotrophic hormone; MC2R, melanocortin-2 receptor; CORT, corticosterone; GFAP, glial fibrillary acidic protein; Iba-1, Ionized calcium binding adaptor molecule 1; iNOS, inducible nitric oxide synthase; Cox-2, cyclooxygenase-2; MIP-1, macrophage inflammatory proteins-1; IL-1 α , interleukin 1 alpha; LIX, lipopolysaccharide-induced CXC chemokine; IGFBP-1, insulin-like growth factor-binding protein-1; ERK, extracellular signal-regulated kinases; CREB, cAMP response element binding protein; NeuN, neuronal nuclear antigen; DCX, doublecortin.

Supplementary figure 2. Effects of DNCB on cutaneous injury and expression of CRH, CRHR1, and CORT in skin. Cutaneous injury was measured by hematoxylin and eosin staining and LDH (A and B). The levels of CRH and CRHR1 (C), and CORT (D), were quantified by immunofluorescence or ELISA kit. Representative photomicrographs are shown (A). Values are means $\pm$ standard error of the mean. *P<0.05 and ***P<0.001 compared with the control group; ###P<0.001 compared with the 2% DNCB-alone group.

Supplementary figure 3. Effects of DNCB on expression of CORT in the brain. The levels of hypothalamic CORT (A), hippocampal CORT (B), and cerebral cortical CORT (C) were measured by ELISA kit. Values are means $\pm$ standard error of the mean. *P<0.05, **P<0.01, and ***P<0.001 compared with the control group; ###P<0.001 compared with the 2% DNCB-alone group.

Supplementary figure 4. Effects of DNCB on neuronal damage in the hippocampus.

Neuronal damage was measured with NeuN immunohistochemistry and Nissl staining in the hippocampus.

Supplementary figure 5. A network of functional relationships generated from proteins using the KEGG pathway. Effects of DNCB on cytokine-cytokine receptor interaction, IL-17 signaling pathway, chemokine signaling pathway, and its related proteins in hippocampus.

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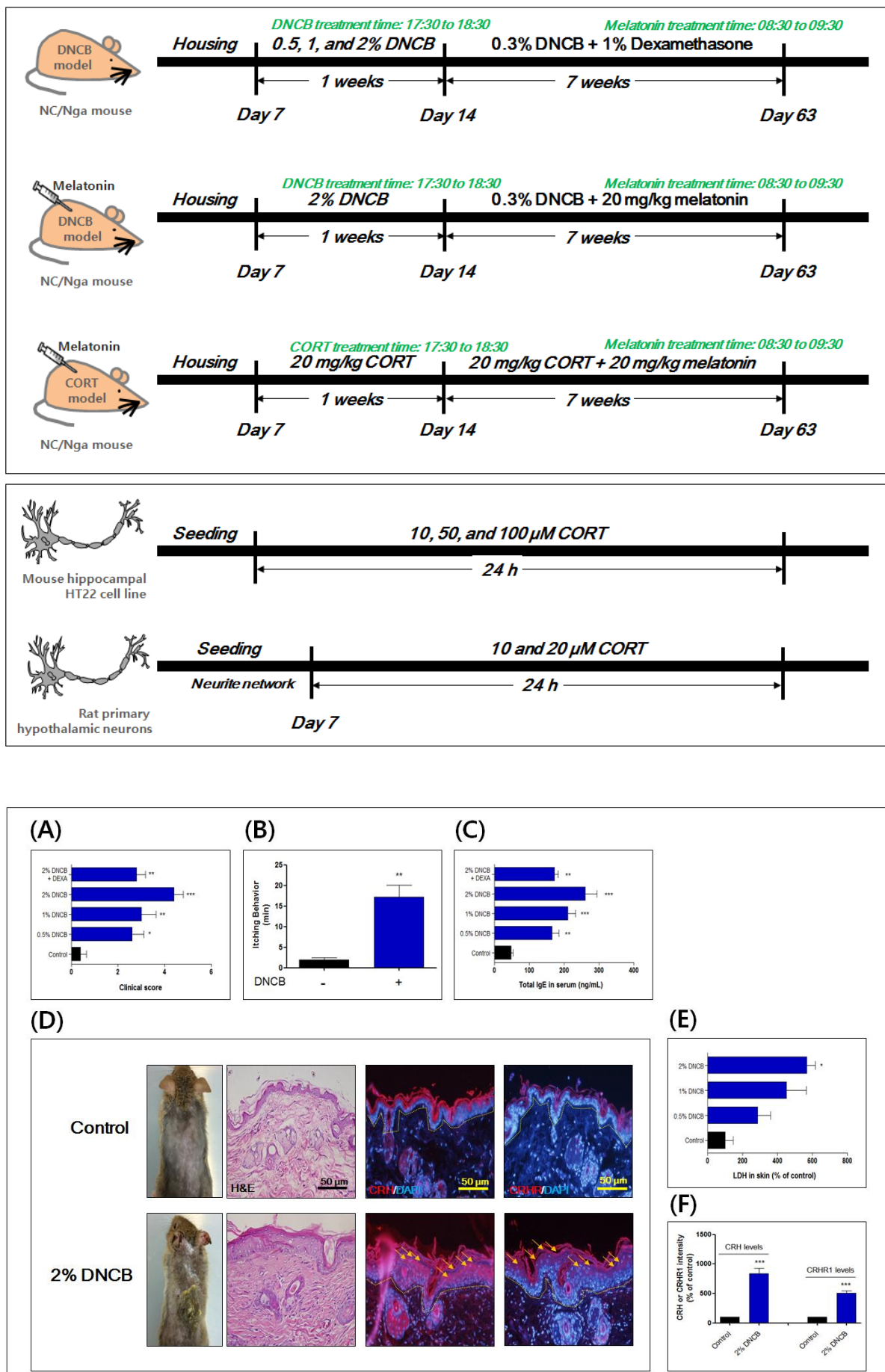
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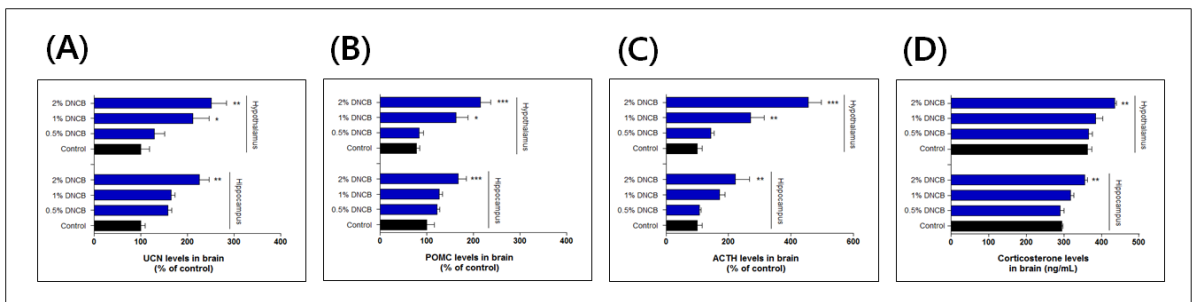
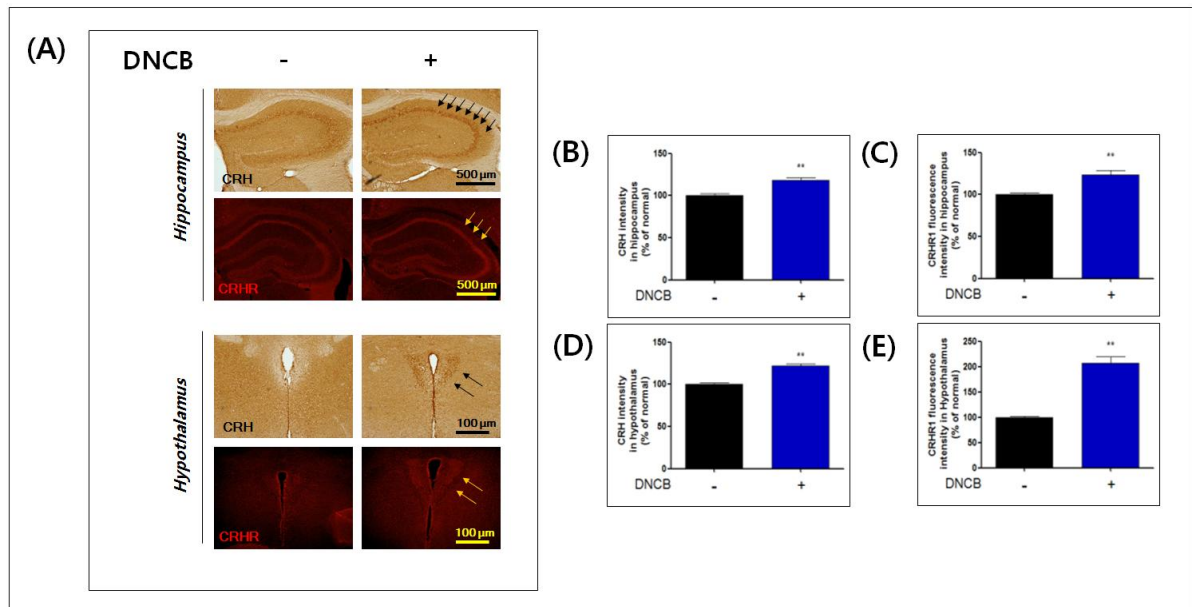
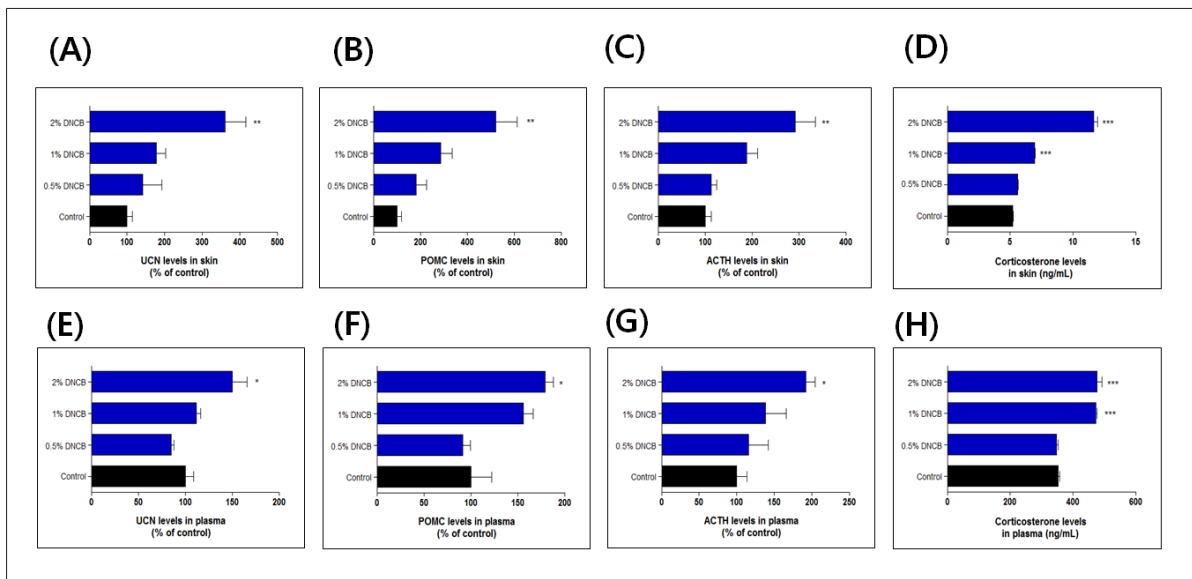
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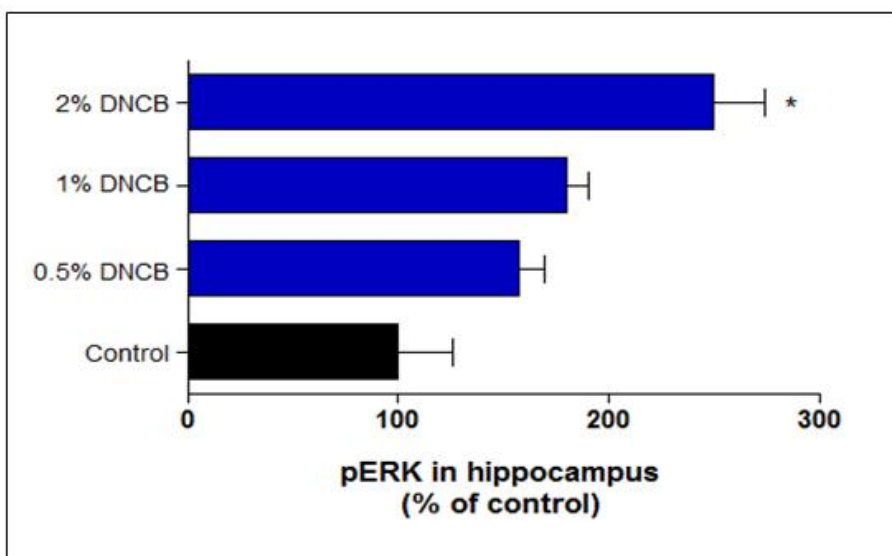
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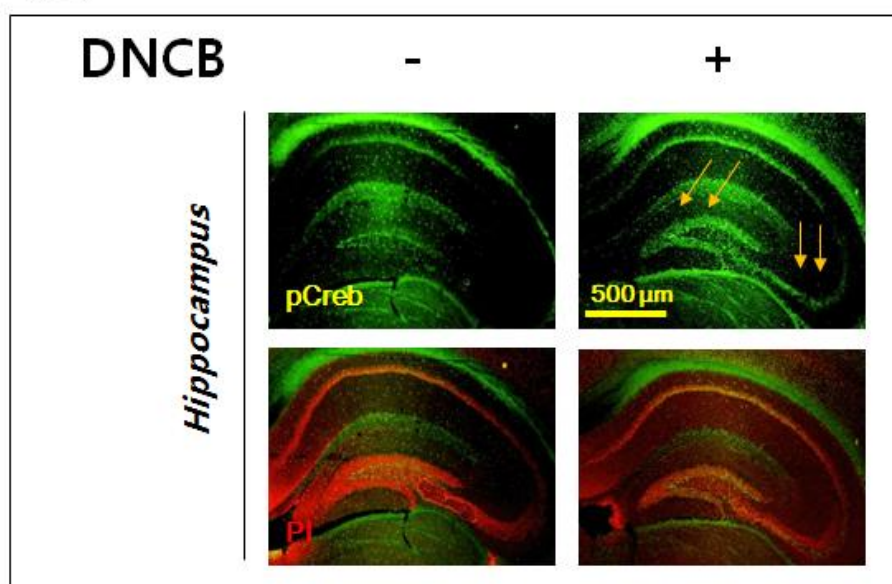




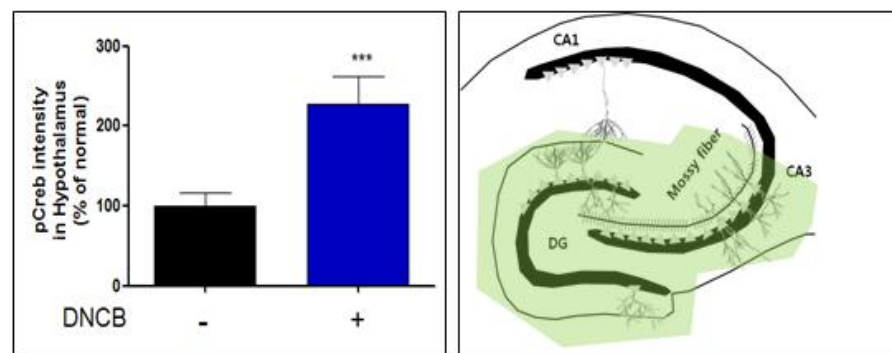
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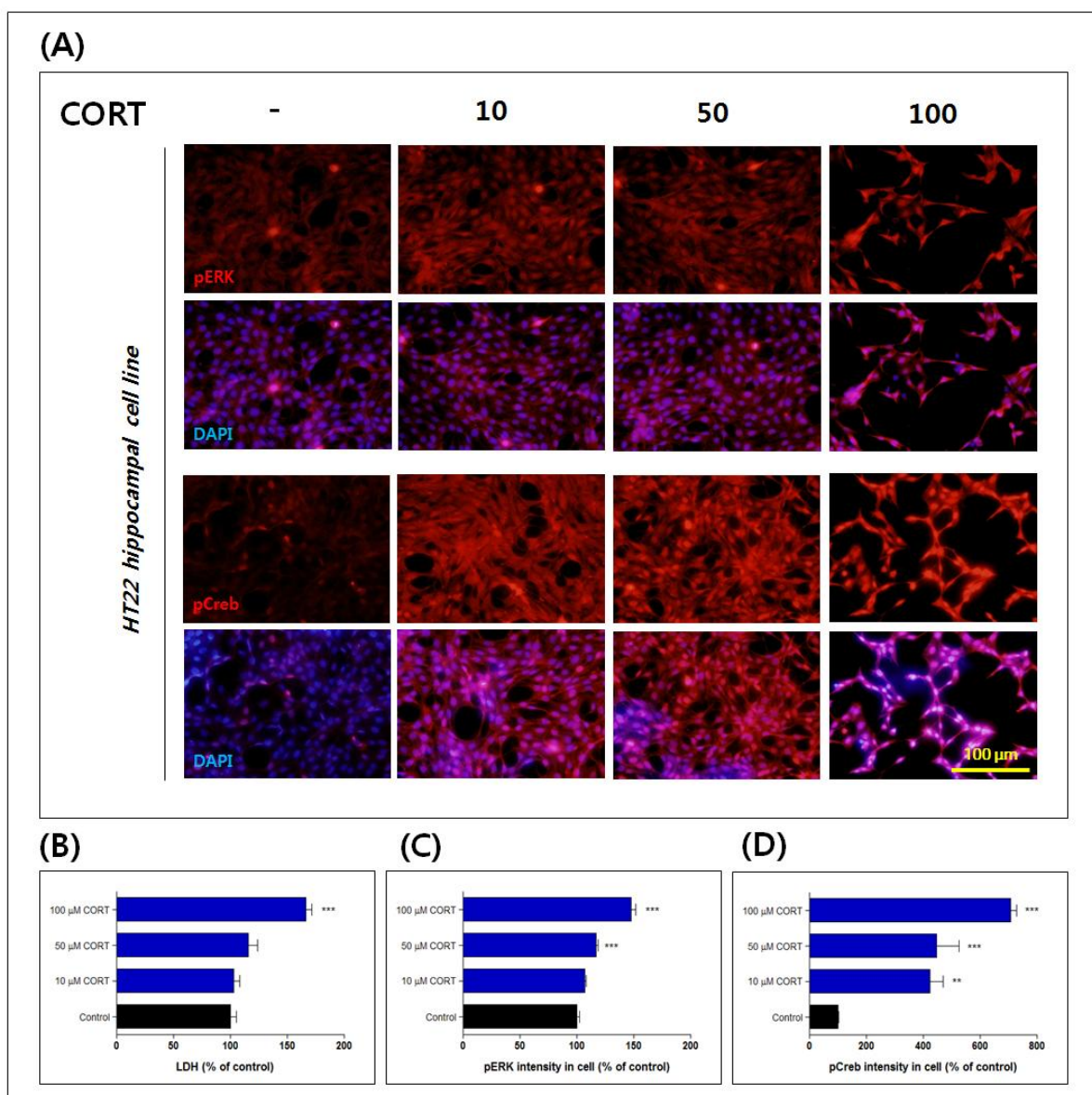


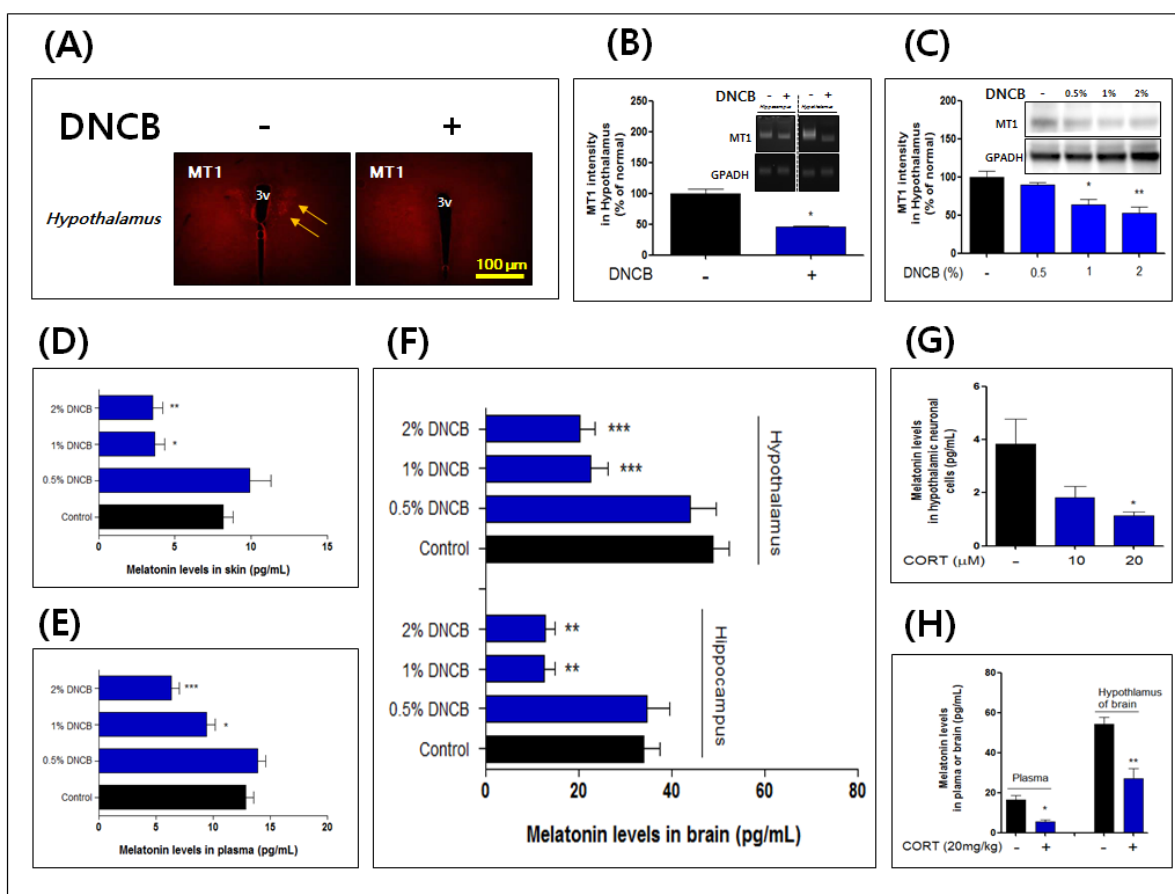
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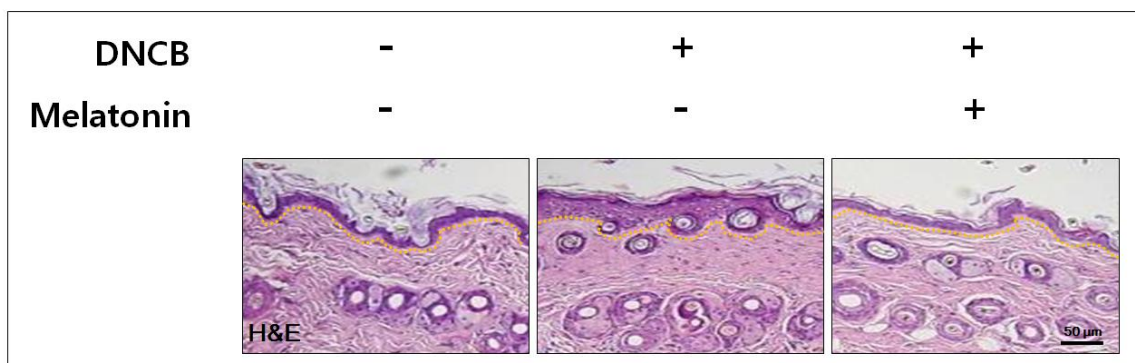
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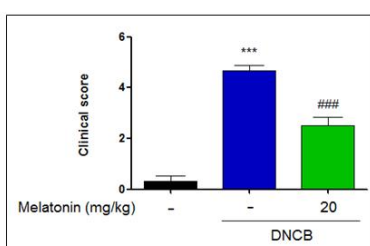




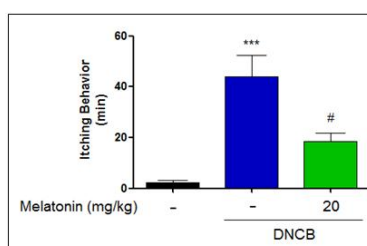
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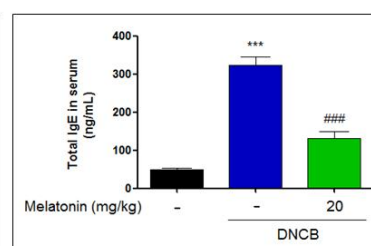
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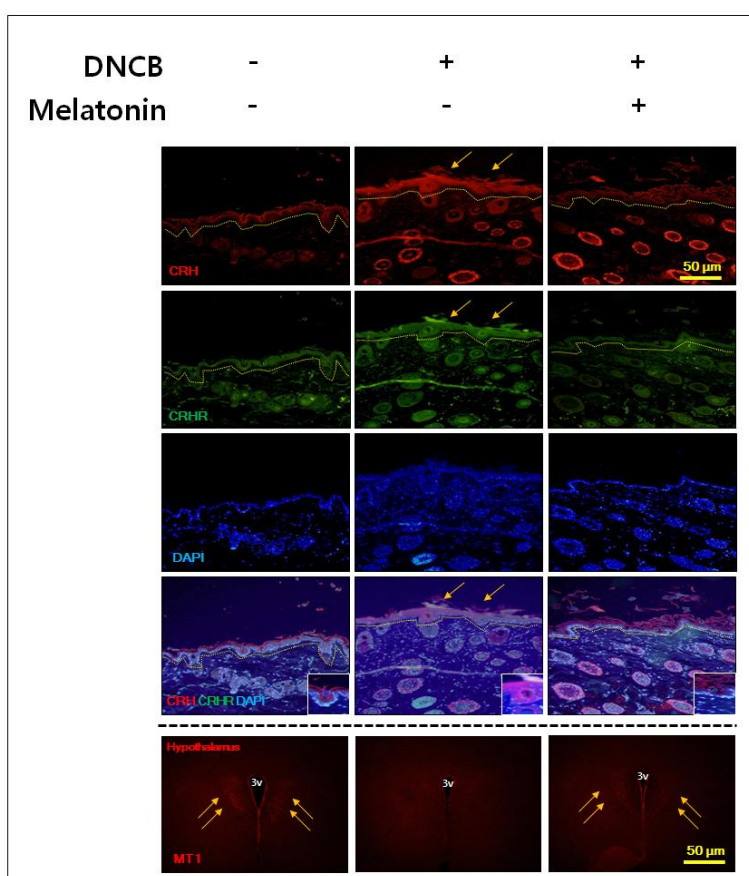
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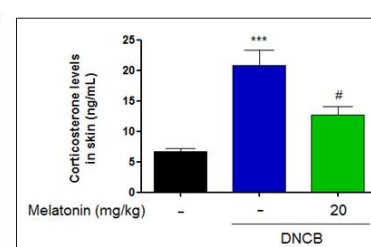
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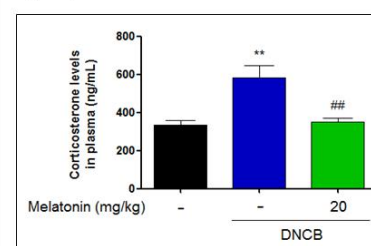
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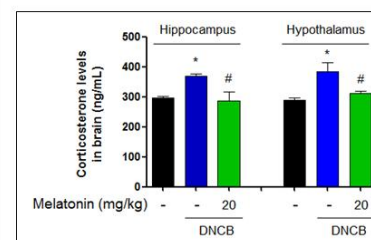
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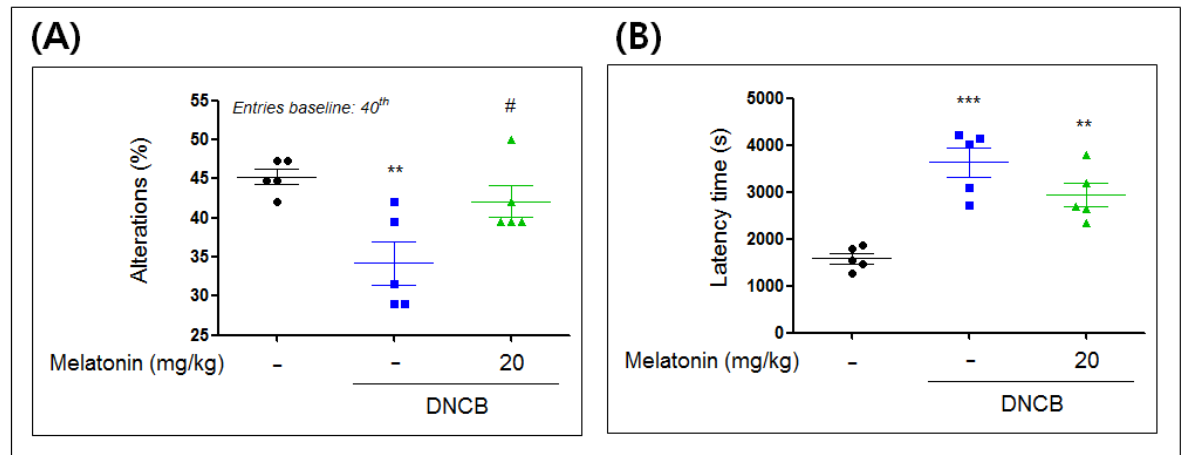
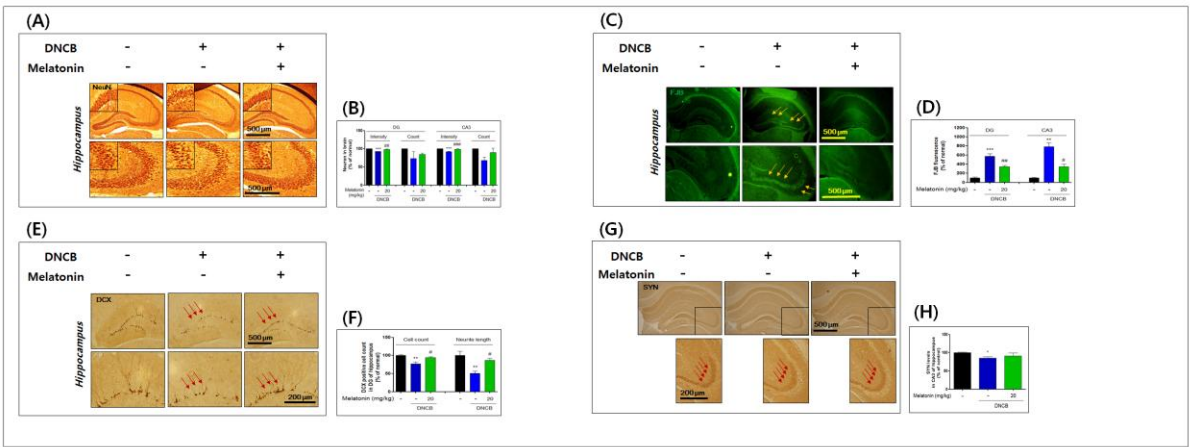
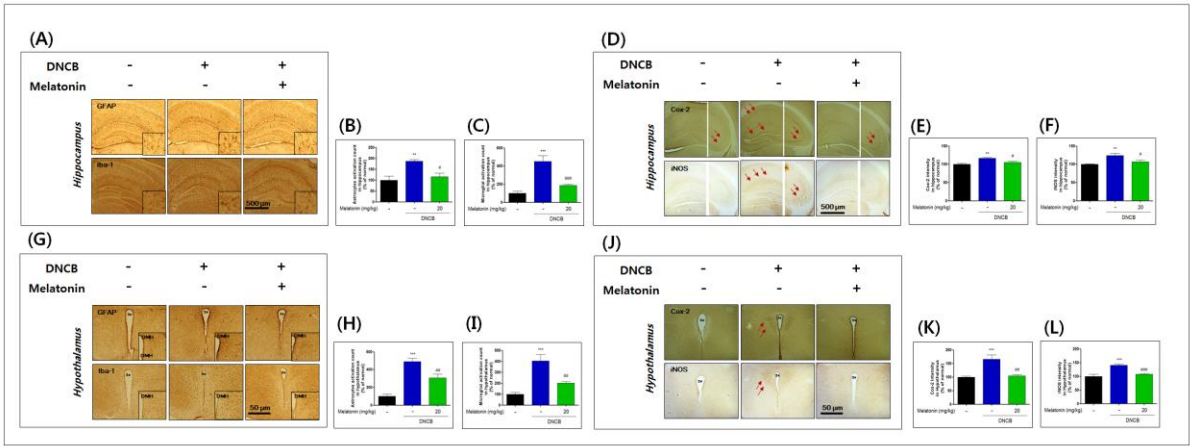


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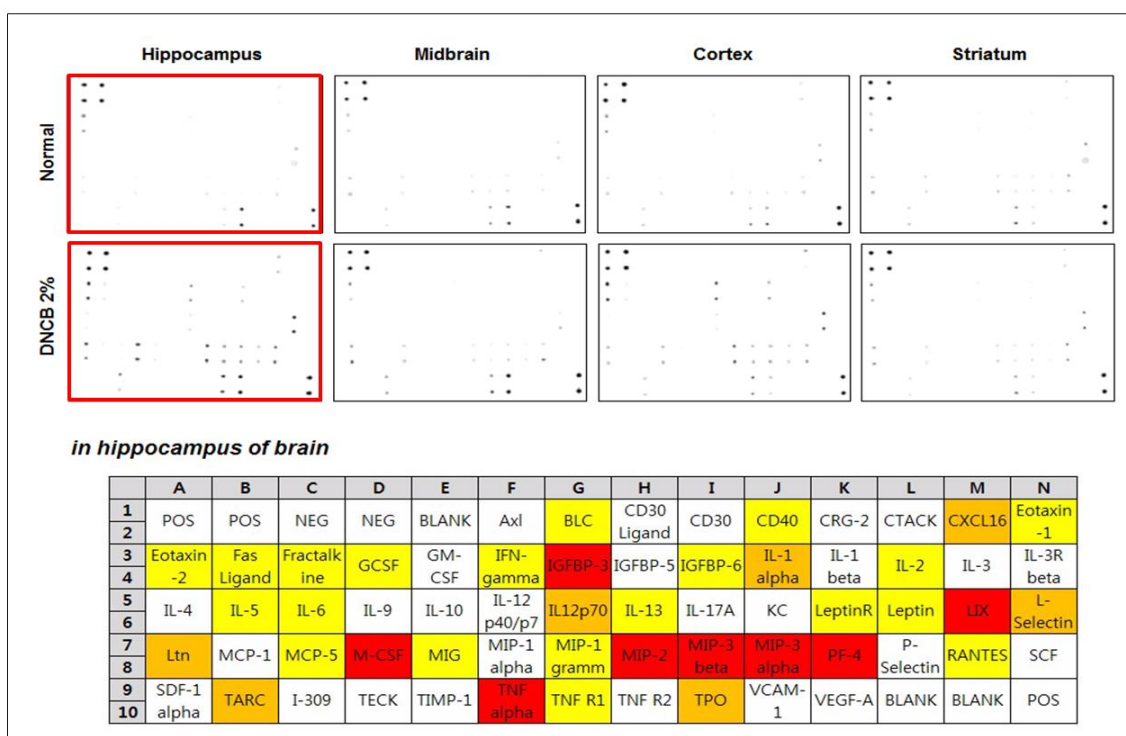


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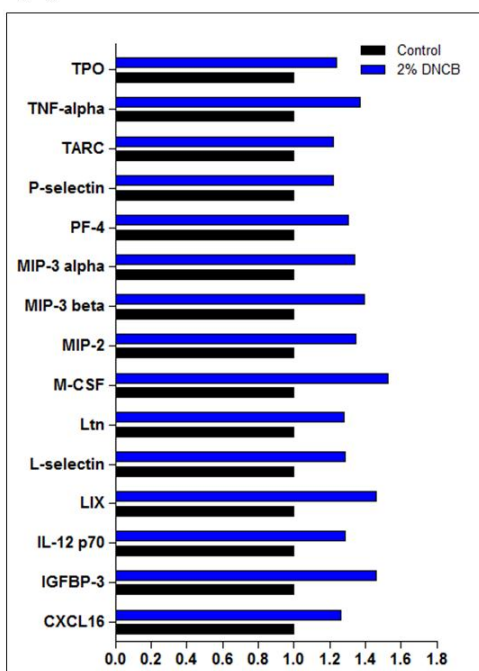




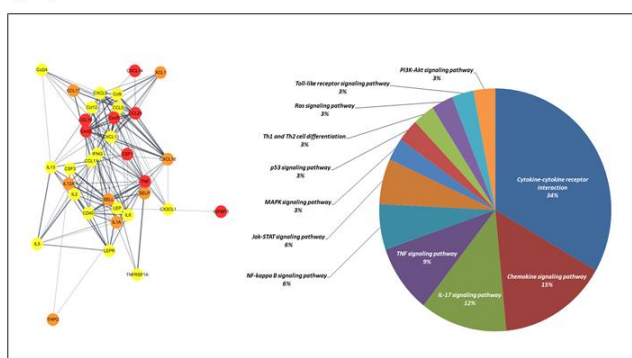
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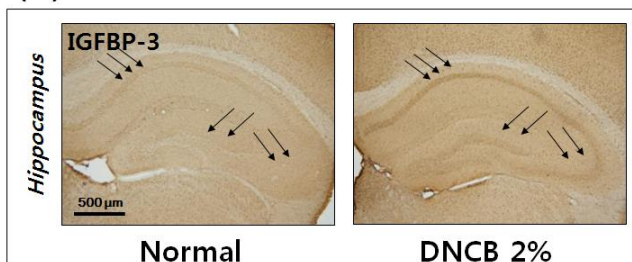
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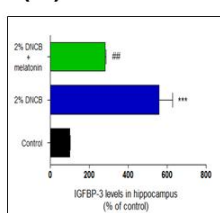
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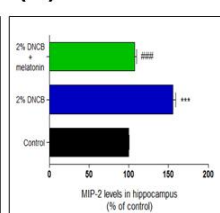
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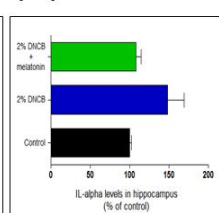
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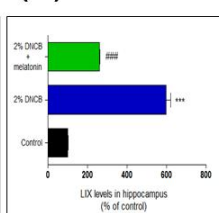
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(H)



(I)

