Upregulation of calcitonin gene-related peptide, neuronal nitric oxide synthase, and phosphorylated extracellular signal-regulated kinase 1/2 in the trigeminal ganglion after bright light stimulation of the eye in rats

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(Received January 27, 2018; Accepted April 6, 2018)

Abstract: Bright light stimulation of the eye activates trigeminal subnucleus caudalis (Vc) neurons in rats. Sensory information is conveyed to the Vc via the trigeminal ganglion (TG). Thus, it is likely that TG neurons respond to photic stimulation and are involved in photic hypersensitivity. However, the mechanisms underlying this process are unclear. Therefore, the hypothesis in this study is bright light stimulation enhances the excitability of TG neurons involved in photic hypersensitivity. Expressions of calcitonin gene-related peptide (CGRP) and neuronal nitric oxide synthase (nNOS) were significantly higher in TG neurons from 5 min to 12 h after photic stimulation of the eye. Phosphorylation of extracellular signal-regulated kinase 1/2 (pERK1/2) was enhanced in TG neurons within 5 min after photic stimulation, while pERK1/2 immunoreactivity in satellite glial cells (SGCs) persisted for more than 12 h after the stimulus. Activation of SGCs was observed from 5 min to 2 h. Expression of CGRP, nNOS, and pERK1/2 was observed in small and medium TG neurons, and activation of SGCs and pERK1/2-immunoreactive SGCs encircling large TG neurons was accelerated after stimulation. These results suggest that upregulation of CGRP, nNOS, and pERK1/2 within the TG is involved in photic hypersensitivity.

Keywords: photosensitivity; trigeminal ganglion; satellite glial cells; CGRP; nNOS; pERK1/2.

Introduction

Photic sensitivity is a common symptom of many diseases, including migraine (1), epilepsy (2), autism spectrum disorder (3), panic disorder (4), fibromyalgia (5), post-traumatic brain injury (6), and dry eye (7). Exacerbation of headache by light is well-known not only in migraine but also in meningitis, subarachnoid hemorrhage, and concussion (8-11). Furthermore, in 1997, a Japanese television cartoon caused seizures induced by flashes of light, even in healthy children, an event referred to as the “Pocket Monsters (Pokemon) incident.”
Neuronal signaling, as a second messenger. NO is generated from L-arginine by nitric oxide synthases (NOSs). Neuronal NOS (nNOS), an isoform of NOS that is important in the signaling of sensory neurons, including nociceptive neurons (31-33). CGRP promotes NO synthesis and release, and vice versa (34-37). During pain, phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and activation of SGCs are promoted by CGRP and NO (27,38) and suppressed by CGRP receptor antagonist administration to the TG (27). ERK1/2, a mitogen-activated protein kinase (MAPK), is phosphorylated within a few minutes after noxious stimulation of peripheral tissues (39) and is involved in initiating and maintaining nociceptive responses in sensory ganglion neurons and SGCs (27,40). Activated SGCs expressing glial fibrillary acidic protein (GFAP) are implicated in the modulation of nociceptive neuron excitability (41,42).

Bright light stimulation of the eye activates Vc neurons via the TG in rats (20,43), and Vc neuronal activity is suppressed by intravitreal injection of a NOS inhibitor (44). This suggests that activation of Vc neurons by bright light stimulation is mediated via TG neurons. Studies have found that a subpopulation of Vc neurons encodes luminosity by increasing response duration and decreasing response latency during an increase in stimulus light intensity (20,44); however, the time course of changes in the expression of molecules in the TG after bright light stimulation is not known. The present study used immunohistochemical techniques to determine if bright light stimulation of the eye time-dependently modulates expressions of CGRP, nNOS, phosphorylated ERK1/2 (pERK1/2), and GFAP in the TG.

Materials and Methods

Animals

The Animal Experimentation Committee of Nihon University approved the animal protocols (AP15D017). All experiments were conducted according to the guidelines of the International Association for the Study of Pain (PHS Low 99-158, revised 2002). Adult male Sprague-Dawley rats weighing 200-350 g (n = 21, Japan SLC, Hamamatsu, Japan) were used. Rats were housed with free access to food and water. Cages remained in a climate- and light-controlled environment (23°C, 12/12-h light/dark cycle with lights turned on at 7:00 AM) for at least 5 days before the experiment. All efforts were made to minimize animal suffering and the number of animals used.

Bright light stimulation and experimental design

All experiments were performed under dim ambient light conditions (<1 lux). Rats were anesthetized initially with intraperitoneal administration of saline solution mixed with 2.5 mg/kg butorphanol (Meiji Seika Pharma, Tokyo, Japan), 0.375 mg/kg medetomidine (Zenoaq, Koriyama, Japan), and 2.0 mg/kg midazolam (Sandoz, Tokyo, Japan), after which they were placed on a warm mat (37°C). White photic stimulation, hereafter referred to as “bright light stimulation”, was delivered from a thermal-neutral fiber optic source (20,000 lux) positioned 5 cm from the left ocular surface for 30 min (30 s ON, 30 s OFF) (17). The ocular surface was kept moist with artificial tears throughout the bright light stimulation and...
while the rats were under anesthesia.

Two experimental designs were used in this study. Design 1 (Fig. 1A) assessed the effects of a single episode of bright light stimulation. At 5 min, 2 h, and 12 h after light stimulation, rats were perfused (n = 3 in each group). Design 2 (Fig. 1B) assessed the effect of bright light stimulation repeated daily for 3 days (1/day × 3 days). At 5 min, 2 h, and 12 h after bright light stimulation on day 3, rats were perfused (n = 3 in each group). Control rats did not receive bright light stimulation (n = 3).

**CGRP, nNOS, pERK1/2, and GFAP immunohistochemistry**

Rats were transcardially perfused with saline followed by cold fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under sodium pentobarbital anesthesia (100.0 mg/kg, i.p.). After perfusion, TGs ipsilateral to the eye receiving bright light stimulation were dissected and immersed in the same fixative as above for 24 h at 4°C and then kept in 0.01 M phosphate-buffered saline (PBS) containing 20% sucrose (w/v), for cryoprotection (12 h). The specimens were then embedded in Tissue Tek (Sakura Finetek, Torrance, CA, USA), and 10-μm sections were cut on the horizontal plane along the long axis of the TG. Every 15th section was thaw-mounted on an MAS-GP microslide glass (Matsunami, Kishiwada, Japan) and dried overnight at room temperature.

Next, sections were incubated with rabbit anti-CGRP polyclonal antibody (1:500, Sigma-Aldrich, C8198, lot #106K4761, St. Louis, MI, USA), rabbit anti-phospho-p44/42 MAPK polyclonal antibody (1:300, Cell Signaling Technology, #9101, Beverly, MA, USA), or mouse anti-GFAP monoclonal antibody (1:800, Millipore AB360, lot #2580636) diluted with 0.01 M PBS containing 4% normal goat serum and 0.3% Triton X-100 (Sigma-Aldrich) for 24 h at 4°C. After washing with 0.01 M PBS for 30 min, sections were incubated in Alexa Fluor 568 anti-rabbit IgG (1:200 in 0.01 M PBS, Invitrogen, #A11011, Paisley, UK) and Alexa Fluor 488 anti-mouse IgG (1:200 in 0.01 M PBS, Invitrogen, #A11001) for 2 h at room temperature. After rinsing with 0.01 M PBS, sections were coverslipped with mounting medium (Thermo Fisher Scientific, Fremont, CA, USA) and examined under a fluorescence microscope (BZ-9000 system, Keyence, Osaka, Japan). Four sections were chosen from each TG at random, and CGRP-IR, nNOS-IR, pERK1/2-IR (neurons or neurons encircled with pERK1/2-IR cells), GFAP-IR, and negative cells in the ophthalmic nerve region were counted at 20× magnification without prior knowledge of the model rats. In the assessment of neurons encircled with pERK1/2-IR and GFAP-IR cells, the number of TG neurons encircled with pERK1/2 or GFAP-IR cells for more than two thirds of their somata perimeters was counted (SensivMeasure, Mitani, Fukui, Japan) (45). The analyzed area was set to 675 × 565 μm² (e.g., Figs. 2A-B, 3A-B, 4A-B, 5A-B, 6A-B). The relative number of cells was calculated by the following formula: number of CGRP-IR neurons, nNOS-IR neurons, pERK1/2-IR neurons, neurons encircled with pERK1/2-IR cells, or neurons encircled with GFAP-IR cells/total number of TG neurons × 100. The size of immunoreactive cells was also measured (SensivMeasure). The following equation calculated the percentage of immunoreactive cells in each class of cell sizes: the number of immunoreactive cells in small, medium, or large-sized neurons/the total number of immunoreactive cells/total number of TG neurons × 100. No specific labeling was observed with the omission of primary antibodies.

**Statistical analysis**

Data are expressed as means ± SEM. The Dunn test was used for individual comparisons after the Kruskal-Wallis test identified significant main effects in the percentage of immunoreactive cells. The relative number of immunoreactive neurons and neurons encircled with immunoreactive cells in each size group (small: <400 μm², medium: 400-800 μm², large: >800 μm²) was assessed by the chi-square test. The significance level was set at P < 0.05 in all tests.
**Results**

**CGRP expression in TG neurons after bright light stimulation**

Numerous CGRP-IR neurons were observed in the TG on day 1, 2 h after bright light stimulation of the eye (Fig. 2A-B).

On day 1, the percentage of CGRP-IR neurons in the ophthalmic nerve region of the TG was significantly higher at 5 min, 2 h, and 12 h after stimulation in rats who had undergone bright light stimulation than in naïve rats (Fig. 2C; naïve = 8.8 ± 2.9%, 5 min = 32.1 ± 2.6%, 2 h = 36.6 ± 2.7%, 12 h = 35.1 ± 6.1%, \( P = 0.0253 \)). Similarly, on day 3, the percentage of CGRP-IR neurons in the ophthalmic nerve region of the TG at 2 h after bright light stimulation was significantly higher than in naïve rats (Fig. 2D; 5 min = 30.2 ± 5.4%, 2 h = 32.6 ± 5.3%, 12 h = 32.2 ± 4.9%, \( P = 0.0564 \)).

To characterize the subset of CGRP-IR neurons after bright light stimulation, the size of CGRP-IR neurons in TG was examined. The frequency of small (<400 μm²), medium (400-800 μm²), and large (>800 μm²) CGRP-IR neurons did not change after bright light stimulation, and most CGRP-IR neurons were classified as small (Fig. 2E).

**nNOS expression in TG neurons after bright light stimulation**

nNOS-IR neurons were observed in the TG on day 1, 2 h after bright light stimulation of the eye (Fig. 3A-B).

On day 1, the percentage of nNOS-IR neurons in the ophthalmic nerve region of the TG was significantly higher at 5 min, 2 h and 12 h after stimulation in rats who had undergone bright light stimulation than in naïve rats (Fig. 3C; naïve = 0.7 ± 0.4%, 5 min = 12.3 ± 1.4%, 2 h = 12.7 ± 0.7%, 12 h = 8.6 ± 1.8%, \( P = 0.0353 \)). In contrast, on day 3, although the percentage of nNOS-IR neurons in the ophthalmic nerve region of the TG was higher after bright light stimulation, it did not significantly differ between rats who had...
undergone bright light stimulation and naïve rats (Fig. 3D; 5 min = 5.0 ± 2.1%, P = 0.2363; 2 h = 4.5 ± 1.0%, P = 0.2633; 12 h = 7.0 ± 5.0%, P = 0.1478). The size distribution of nNOS-IR neurons did not change after bright light stimulation, and most nNOS-IR neurons were classified as small or medium sized (Fig. 3E).

**pERK1/2 expression in TG neurons after bright light stimulation**

Numerous pERK1/2-IR neurons were observed in TG neurons on day 3, five minutes after bright light stimulation of the eye (Fig. 4A-B).

On day 1, the percentage of pERK1/2-IR neurons in the ophthalmic nerve region of the TG was significantly higher at 5 min after stimulation in bright light-stimulated rats than in naïve rats (Fig. 4C; naïve = 0.8 ± 0.2%; 5 min = 8.3 ± 1.9%, P = 0.0299; 2 h = 2.3 ± 2.1%, P = 0.8178; 12 h = 2.6 ± 0.7%, P = 0.1669). On day 3, the percentage of pERK1/2-IR neurons in this region was significantly higher at 5 min after stimulation in the bright light-stimulated rats than in naïve rats (Fig. 4D; 5 min = 19.5 ± 4.8%, P = 0.0070; 2 h = 1.1 ± 0.3%, P = 0.5985; 12 h = 0.7 ± 0.3%, P = 0.9214), and the percentage of pERK1/2-IR neurons was significantly higher in the TG at 5 min after bright light stimulation than at 2 h and 12 h after stimulation (Fig. 4D; 2 h: P = 0.0299; 12 h: P = 0.0052). The most pERK1/2-IR neurons were classified as small or medium sized (Fig. 4E).

**pERK1/2 expression in SGCs encircling TG neurons after bright light stimulation**

The number of TG neurons encircled with pERK1/2-IR cells gradually increased on day 3, starting at 5 min after bright light stimulation (Fig. 5A-B).

On day 1, the percentage of TG neurons encircled with pERK1/2-IR cells in the ophthalmic nerve region of the TG was significantly higher at 2 h and 12 h after stimulation in bright light-stimulated rats than in naïve rats (Fig. 5C; naïve = 2.3 ± 0.6%; 5 min = 2.6 ± 0.7%, P = 0.5537; 2 h = 31.0 ± 13.9%, P = 0.0353; 12 h = 42.1
± 10.1%, $P = 0.0038$), and the percentage of TG neurons encircled with pERK1/2-IR cells was significantly higher at 12 h after than at 5 min after bright light stimulation in light-stimulated rats (Fig. 5C; $P = 0.00213$). Similarly, on day 3, the percentage of TG neurons encircled with pERK1/2-IR cells in this region was significantly higher at 2 h and 12 h after stimulation in bright light-stimulated rats than in naïve rats (Fig. 5D; 5 min = 16.7 ± 2.4%, $P = 0.1882$; 2 h = 29.4 ± 4.2%, $P = 0.0719$; 12 h = 49.7 ± 7.1%, $P = 0.0016$). The frequencies of small, medium, and large neurons encircled with pERK1/2-IR cells did not differ between bright light-stimulated and naïve rats, and most neurons encircled with pERK1/2-IR cells were large neurons (Fig. 5E).

**GFAP expression in the TG after bright light stimulation**

TG neurons encircled with GFAP-IR cells were observed from 5 min after bright light stimulation of the eye on day 1 (Fig. 6A-B).

On day 1, the percentage of TG neurons encircled with GFAP-IR cells in the ophthalmic nerve region of the TG was significantly higher from 5 min to 2 h after bright light stimulation in bright light-stimulated rats than in naïve rats (Fig. 6C; naïve = 0.7 ± 0.1%; 5 min = 12.6 ± 1.7%, $P = 0.0213$; 2 h = 15.0 ± 2.8%, $P = 0.0070$; 12 h = 9.8 ± 3.9%, $P = 0.0757$). On day 3, the percentage of TG neurons encircled with GFAP-IR cells was also significantly higher at 5 min after bright light stimulation in bright light-stimulated rats than in naïve rats (Fig. 6D; 5 min = 11.0 ± 0.9%, $P = 0.0299$; 2 h = 10.4 ± 3.4%, $P = 0.0654$; 12 h = 9.8 ± 5.4%, $P = 0.1000$). The size of TG neurons encircled with GFAP-IR cells did not differ between bright light-stimulated rats and naïve rats, and most neurons encircled with GFAP-IR cells were large neurons (Fig. 6E).

**Percentage of immunoreactive cells at different time points after bright light stimulation**

Change over time in the percentage of immunoreactive cells was examined in the TG after bright light stimulation (Fig. 7A). There were no differences in the percentages of CGRP-, nNOS- or pERK1/2-IR in the TG neurons, or in the percentages of pERK1/2- and GFAP-IR in SGCs between day 1 and day 3 at any time point after bright light stimulation.

**Discussion**

The main findings of this study are as follows: (1) Expressions of CGRP and nNOS were persistently enhanced in TG neurons from 5 min to 12 h after bright light stimula-
tion (2). Phosphorylation of ERK1/2 in TG neurons was detected at 5 min after bright light stimulation, while pERK1/2 expression in SGCs gradually increased from 2 to 12 h (3). SGC activation was observed from 5 min to 2 h after bright light stimulation (4). Upregulation of CGRP, nNOS, and pERK1/2 was observed primarily in small neurons, and pERK1/2 and GFAP-IR were found in SGCs encircling large TG neurons (6). The sizes of CGRP-IR, nNOS-IR, and pERK1/2-IR neurons and neurons encircled with pERK1/2-IR and GFAP-IR cells were unchanged after bright light stimulation.

Frequent bright light stimulation of the eye can induce ocular pain and headache, and the mechanisms underlying such pain are thought to be higher-order processing in the thalamus and somatosensory cortex, or trigeminal nucleus caudalis neurons receiving nociceptive information from the eye (19,20,46,47). Bright light also alters the descending pain control system by modulating ON-cell and OFF-cell activities in the rostral ventromedial medulla (5). A recent study reported that TG neurons in humans and mice express melanopsin. Melanopsin-IR TG neurons respond to light and co-express CGRP (28). CGRP is an extremely potent vasodilator and multifunctional regulatory neuropeptide that is critical in the pathogenesis of neuropathic (27) and inflammatory pain (48) and headache (49). CGRP is synthesized and released from small TG neurons (25), and CGRP-IR neurons innervate intracranial blood vessels (24). Nitric oxide, a diffusible molecule generated by NOS, is also an endogenous substance and another key mediator in migraine and other primary headaches (50). Neuronal NOS is present in small nerve fibers and the ramifications running along branches of the middle meningeal artery (37). In the TG, NOS-IR is mainly observed in small to medium neurons (51), some of which also show CGRP-IR (52). CGRP promotes NO synthesis and release, and vice versa (34-37). Nitroglycerin infusion increases the number of CGRP-IR and NOS-IR TG neurons in the TG after 4-6 h (52), and intravitreal injection of NOS inhibitors suppresses light-evoked neuronal activity in the Vc and, within 10 min, increases ocular blood flow caused by bright light stimulation (44). Bright light stimulation of the eye immediately upregulated CGRP and NOS production in the TG in this study. Past and present evidence suggests that CGRP and NOS are involved in enhancing the trigeminovascular interaction after bright light stimulation, which results in photic hypersensitivity associated with frequent bright light stimulation of the eye.

CGRP receptors are present on large TG neurons not expressing CGRP and SGCs (53). CGRP could cause activation of SGCs via phosphorylation of ERK1/2 in SGCs (27). Phosphorylation of ERK is important in noxious signaling (39,40). Levels of pERK increased in TG neurons and SGCs from 15 min to 2 h after injection of an NO donor into the temporomandibular joint and returned to basal levels at 24 h (38). After nerve injury, CGRP is markedly upregulated in small primary neurons, and phenotypic switch occurs in medium and large neurons (27,54). After nerve crush injury, more large TG neurons encircled by GFAP-IR SGCs than small neurons were observed (45). In contrast, the size distribution of CGRP-, Substance P-, galanin- and P2X3-IR neurons (which are normally expressed in small primary neurons) were unchanged under inflammatory conditions (55,56). While phosphorylation of ERK1/2 underlying neuropathic pain only occurred in SGCs (27), it occurred in both SGCs and neurons during inflammation (38,57). In this study, the size distribution of CGRP-, nNOS- and pERK1/2-IR neurons and neurons encircled with pERK1/2- and GFAP-IR SGCs did not change, and phosphorylation of ERK1/2 occurred in both neurons and SGCs. Sunlight affects the immune system, leading to beneficial effects for human health (e.g., reductions in autoimmune diseases and cancers) (58-60), and is involved in T cell motility (61). After bright light stimulation of the eye, CGRP, NOS, and pERK1/2 were immediately upregulated in small neurons, and phosphorylation of ERK, which transmits noxious signals, was not prolonged. CGRP and NOS might induce activation of SGCs surrounding large neurons in bright light-stimulated rats. Taken together, our findings suggest that bright light stimulation of the eye enhances neuronal and glial cell activation in the TG, because of accelerated immune function and neuron-glia interactions in the TG.

Because of the absence of electrophysiological data, it could not be determined the precise neuronal activity of TG neurons after bright light stimulation. Phosphorylation of ERK, which is considered a marker of neuronal excitability in TG neurons, was observed 5 min after bright light stimulation. CGRP and NOS were also upregulated within 5 min, and upregulation of these molecules persisted for longer than 12 h. Furthermore, activation of SGCs was observed at 5 min after bright light stimulation, and ERK phosphorylation in SGCs occurred from 2 to 12 h after stimulation. These findings suggest that bright light stimulation can activate TG neurons and SGCs immediately after the stimulus and that these effects may persist for longer than 12 h.

Bright light is thought to induce abnormal responses in the peripheral and central nervous system. However, it is also a useful therapy for sleep disorders and major
depression, except when these conditions are related to dementia (15,62). Light activates ipRGCs to release glutamate in the hypothalamic suprachiasmatic nucleus (SCN) through a monosynaptic pathway, the retinohypothalamic tract (63-65). The activated SCN modulates mood (66,67), sleep (68,69), circadian rhythms (69), and hypothalamic-pituitary axis activity (70,71). Intrinsically photosensitive RGCs in the iris contribute to trigeminal afferents detouring to the optic nerve (72,73). Light therapy for treatment of depression also targets various brain regions and synapses (74,75). There are no residual effects or tolerance to light therapy, although headache, eye strain, and hypomania are known side effects (66).

Furthermore, the present time course changes were similar after exposure to bright light stimulation for 1 day and 3 consecutive days in all molecules (Fig. 7A), which suggests that the effects of bright light stimulation evanesce and are reversible. A light intensity of 2,000-10,000 lux was used for light therapy in most human studies (62). In most animal studies, however, 500-50,000 lux was used to activate light-responsive neurons in the central nervous system: the posterior thalamus (19), rostral ventromedial medulla (5), and Vc (20,43). In this study, an intensity of 20,000 lux was able to modulate the peripheral nervous system, and the accumulation of daily bright light exposure was not immense. Furthermore, green light is better than white, blue, or red light in attenuating headache in humans (76) and neuropathic pain in rats (77). Because there is no consensus regarding light therapy, future studies need to clarify the mechanisms and neuronal pathways involved in light perception, as well as optimal treatment duration, light color, and intensity.

Acknowledgments
The authors thank the undergraduate students of Nihon University School of Dentistry in the “Approach to Basic Research” course-Reina Kanai, Mami Teranishi, Yuuto Suzuki (2014), Ryo Tanaka, Takahiro Togashi (2015), Yoshihiro Momose, Maimu Kanari (2016), Masaoki Hikosaka, Yuma Isa, and Konoka Takasaki (2017)-for their contributions to this project. This work was supported in part by JSPS KAKENHI grant number 17K11855 to AK, by AMED grant number 17e0610012h0001, by research grants from the Sato and Uemura Funds of Nihon University School of Dentistry, and by a grant from the Dental Research Center of Nihon University School of Dentistry.

Conflict of interest
The authors report no financial or other conflict of interest.

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