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$GABA_B$ RECEPTORS ACCENTUATE NEURAL EXCITATION CONTRAST IN RAT INSULAR CORTEX

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Abstract—Synaptic transmission mediated by metabotropic GABA receptors, $GABA_B$ receptors, regulates physiological functions of cerebrocortical local circuits. It is, however, still unknown how $GABA_B$ receptors regulate excitatory propagation at more macroscopic level. We performed *in vivo* optical imaging to investigate the spatio-temporal profiles of $GABA_B$ receptor-mediated regulation of excitatory propagation in anesthetized rat insular cortex (IC). Repetitive electrical stimulation (a sequence of 10 pulses at 50 Hz) of the dysgranular IC (DI), a part of gustatory cortex (GC), elicited excitatory propagation along the rhinal fissure. Tonic activation of $GABA_B$ receptors by application of baclofen suppressed the optical signal amplitude to the early pulses in the sequence (first to third stimuli), typically in the rostral GC (rGC). In contrast, optical signal amplitude to later pulses was enhanced by baclofen in both the rGC and caudal GC (cGC). Baclofen reduced the area of excitation during the early pulses in the sequence but not during later pulses. Application of CGP 52432, which blocked $GABA_B$ receptor-mediated tonic and phasic inhibition, slightly suppressed optical responses to early pulses (though not to the first pulse), whereas it enhanced responses to later pulses, especially in the dorsolateral orbital cortex (DLO). Decay amplitude of the response to the first pulse was reversed to a large rise in amplitude by the $GABA_A$ receptor antagonist bicuculline. The decay amplitude was enhanced by CGP 52432 and reversed to a small rise by baclofen. This suggests that $GABA_B$ receptor activation reduced postsynaptic $GABA_A$ receptor activation indirectly via inhibition of presynaptic GABA release. Optical responses induced by DLO stimulation were reduced by pre-stimulation of the cGC 180 ms before DLO stimulation, which was blocked by CGP 52432. These results suggest that tonic and phasic activation of $GABA_B$ receptors cooperatively enhances the contrast of neural excitation at a level of millimeters.

Key words: optical imaging, voltage-sensitive dye, taste, $GABA_B$, cerebral cortex.

In the cerebral cortex, GABA plays a crucial role in regulating neural information processing. $GABA_B$ receptors are metabotropic receptors that modulate neural activities via several mechanisms, including presynaptic inhibition of excitatory postsynaptic currents (EPSC) (Mintz and Bean, 1993; Gil et al., 1997; Sakaba and Neher, 2003), induction of slow inhibitory postsynaptic potentials (IPSPs) or currents (Connors et al., 1988; Nathan et al., 1990; Lüscher et al., 1997; Chalifoux and Carter, 2010) and tonic inhibition mediated through extrasynaptic receptors (Wang et al., 2010). Significant reduction of GABAergic inhibition causes profound dysfunction in the cerebral cortex, such as epileptiform activity (Treiman, 2001; Teichgräber et al., 2009) and disturbance of neural plasticity (Hensch et al., 1998).

Excitatory propagation revealed by optical imaging, using a voltage-sensitive dye, reflects membrane potentials. Therefore, optical imaging is a potent tool to macroscopically analyze spatiotemporal profiles of neural excitability (Tominaga et al., 2000; Berger et al., 2007; Chemla and Chavane, 2010). Compared to imaging in brain slice preparations, *in vivo* optical imaging has a potential advantage for identifying functional roles of GABAergic receptors; this relates particularly to excitatory propagation beyond the boundaries of cortical regions, due to almost complete preservation *in vivo* of neural circuits. Blockade of $GABA_A$ receptors induces prolonged excitatory propagation, with robust increases in amplitude of responses to intrinsic (Horikawa et al., 1996) or electrical stimulation (Tanifuji et al., 1994; Fujita et al., 2010). Miyakawa et al. (2003) reported that acute, spontaneous epileptiform discharges are induced by bicuculline, a $GABA_A$ receptor antagonist. Compared to $GABA_A$ receptor-mediated regulation of cortical excitation, far less is known about how $GABA_B$ receptor-mediated inhibition controls excitatory propagation.

The insular cortex (IC), which is located on the dorsal bank of the rhinal fissure, is divided into three areas based on cytoarchitecture: the dorsal granular region (GI), the intermediate dysgranular region (DI) and the ventral agranular region (AI). These subregions are considered to process different sensory modalities, including gustation,

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Abbreviations: AI, agranular insular cortex; cGC, caudal part of gustatory cortex; DI, dysgranular insular cortex; DLO, dorsolateral orbital cortex; EPSC, excitatory postsynaptic currents; EPSP, excitatory postsynaptic potential; *F*, intensity of fluorescence; Fr3, frontal cortex (area 3); GC, gustatory cortex; GI, granular insular cortex; IC, insular cortex; IPSP, inhibitory postsynaptic potential; ISI, inter-stimulus interval; LFP, local field potential; LI, localization index; MC, motor cortex; MCA, middle cerebral artery; PPR, paired-pulse ratio; rGC, rostral part of the gustatory cortex; ROI, regions of interest; *S*, area of significant response; SEM, standard error of the mean; SI, primary somatosensory cortex; SII, secondary somatosensory cortex; Var, variance of optical signals.

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visceral sensation, and nociception (Krettek and Price, 1977; Allen et al., 1991; Jasmin et al., 2004). Therefore, IC is considered to be an ideal area to explore GABAergic roles in the control of excitatory propagation between cortical subregions. Previously, we demonstrated that application of bicuculline robustly enhanced the amplitude of excitatory propagation in response to DI stimulation, which, in turn, spread not only in the rostrocaudal but also in the dorsoventral direction (Fujita et al., 2010). Conversely, Ajima et al. (1999) reported that saclofen, a $GABA_B$ receptor antagonist, expanded activation of somatosensory regions, as revealed in an anisotropic manner by intrinsic optical signal imaging. This suggests that $GABA_B$ receptors may play a role in disrupting specific cortico-cortical communication. To explore this possibility, optical imaging in anesthetized rats was performed to examine the effects of the $GABA_B$ receptor agonist baclofen and the $GABA_B$ receptor antagonist CGP 52432 on the excitatory propagation evoked by electrical stimulation of a part of the DI, the gustatory cortex (GC).

EXPERIMENTAL PROCEDURES

Experiments were approved by the Animal Experimentation Committee of Nihon University School of Dentistry and were performed in accordance with institutional guidelines for the care and use of experimental animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Surgical procedures

Five-week old male Sprague–Dawley rats (Sankyo Labo, Tokyo, Japan) weighing 147±3 g (n=47) received atropine methyl bromide (5.0 mg/kg, i.p. injection) and were anesthetized with urethane (1.5 g/kg, i.p. injection). Anesthesia was maintained throughout the experiments by injecting additional urethane. The adequacy of anesthesia was gauged by the absence of the toe pinch reflex. Body temperature was monitored using a rectal probe (BWT-100, Bio Research Center, Tokyo, Japan) and was maintained at approximately 37 °C using a heating pad. Animals received a tracheotomy and intubation, with lidocaine (2% gel) applied to the incisions to ensure complete analgesia. The anesthetized animal was mounted on a custom-made stereotaxic snout frame (Narishige, Tokyo, Japan), which was then tilted 60° laterally to make the left IC accessible to the CCD camera system and electrodes. The left temporal muscle and zygomatic arch were carefully removed, and a craniotomy was performed to expose the IC and the surrounding cortices.

Optical recording

The method of optical imaging using a voltage-sensitive dye was as described previously (Chen et al., 2010; Fujita et al., 2010; Kobayashi et al., 2010), and only a brief account of the methods employed is given here. The voltage-sensitive dye, RH1691 (1 mg/ml, Optical Imaging, NY, USA) in 0.9% saline, was applied to the cortical surface for approximately 1 h; residual dye was rinsed with saline for approximately 30 min, and fluorescent changes of RH1691 were measured using a CCD camera system (MiCAM02, Brainvision, Tokyo, Japan) mounted on a stereomicroscope (Leica Microsystems, Wetzler, Germany). The cortical surface was illuminated through a 632 nm excitation filter and a dichroic mirror using a tungsten-halogen lamp (CLS150XD, Leica Microsystems), and the fluorescent emission was captured through an absorption filter (λ>650 nm long-pass, Andover, Salem, NH, USA). The CCD-based camera had a 6.4×4.8 mm^2 imaging area consisting of 184×124 pixels. To remove signals due to acute bleaching of the dye, the final image was obtained by subtracting an image without stimulation from an image with stimulation. The sampling interval was 4 ms, and the acquisition time was set at 500 ms. Twenty-four consecutive images in response to stimuli were averaged to reduce noise, including interference by heartbeat, breathing and spontaneous neural activity.

Electrical stimulation

A tungsten electrode (impedance=10 MΩ; FHC, Bowdoin, ME, USA) was inserted 0.3 mm from the surface into the cortex for electrical stimulation (100 μs duration, 7 V). In repetitive stimulation experiments, the inter-stimulus interval (ISI) was set at 20 ms, and 5 or 10 pluses were delivered. In paired-pulse stimulation experiments, the ISI was set at 180 ms, when the amplitude of $GABA_B$ receptor-mediated slow IPSPs, evoked by electrical stimulation, reached approximately the maximum in IC pyramidal neurons (data not shown). In some paired-pulse stimulation experiments, stimulation electrodes were set at the caudal part of the gustatory cortex (cGC) and at the dorsolateral orbital cortex (DLO), with DLO stimulation preceding cGC stimulation by 180 ms. An indifference electrode made from AgCl was set at the margin of the skull window. Pulse trains were applied at 30 s intervals.

Field potential recording

To confirm that the responses of optical signals reflected electrical changes, field excitatory postsynaptic potential (EPSP) slopes were recorded using a monopolar tungsten electrode (impedance=250 kΩ) placed caudally relative to the stimulation electrode. Evoked responses were amplified, filtered (band pass: 0.5–1000 Hz; AB-651J, Nihon Koden, Tokyo, Japan), recorded using an interface (Micro 1401 MK2, Cambridge Electronic Design, Cambridge, UK) and stored on a computer hard disk.

Drugs

Atropine methyl bromide, urethane and bicuculline methiodide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Baclofen ((*R*)-4-amino-3-(4-chlorophenyl)butanoic acid) and CGP 52432 (3-[[[(3,4-dichlorophenyl)methyl]amino]propyl] diethoxymethyl)phosphinic acid were purchased from Tocris BioScience (Ellisville, MO, USA). All drugs were dissolved in saline immediately before use. Baclofen (10, 100 μM), CGP 52432 (10 μM) and bicuculline methiodide (10 μM) were applied directly onto the cortical surface for 30 min.

Data analysis

The change in the intensity of fluorescence (ΔF) in each pixel relative to the initial intensity of fluorescence (F) was calculated ($\Delta F/F$). A significant response was defined as a signal exceeding by three-fold the SD of baseline noise. In measurements of amplitude, images of responses were processed with a spatial filter (9×9 pixels) to avoid influence of noise and brain movements per pixel. Optical imaging data were processed and represented using software (Brain Vision Analyzer, Brainvision, Tokyo, Japan).

To quantify the decay speed of the optical signals after repetitive electrical stimuli, the curve of decay after 5 train pluses was fitted exponentially, and the time constant was calculated using OriginPro 8 (OriginLab, Northampton, MA, USA).

To quantify the spatial pattern of the activated area, the relationship of variance of optical signals (Var) and the area of significant response (S; mm^2) was defined as the "localization index (LI)," which was calculated using the following equation:

$$LI = Var/S \quad (1)$$