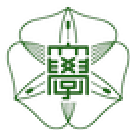

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GABAergic mechanisms for shaping transient visual responses in the mouse superior colliculus

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Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; CGP, CGP52432; GABA, gamma aminobutyric acid; NFV, narrow-field vertical; NMDA, *N*-methyl-D-aspartate; PB, phosphate buffer; PBS, phosphate-buffered saline; PSTH, peristimulus time histogram; SC, superior colliculus; sSC, superficial layer of the superior colliculus; V1, primary visual cortex; WFV, wide-field vertical.

Abstract

An object that suddenly appears in the visual field should be quickly detected and responded to because it could be beneficial or harmful. The superficial layer of the superior colliculus (sSC) is a brain structure capable of such functions, as sSC neurons exhibit sharp transient spike discharges with short latency in response to the appearance of a visual stimulus. However, how transient activity is generated in the sSC is poorly understood. Here, we show that inhibitory inputs actively shape transient activity in the sSC. Juxtacellular recordings from anesthetized mice demonstrate that almost all types of sSC neurons, which were identified by *post hoc* histochemistry, show transient spike discharges, i.e., ON activity, immediately after visual stimulus onset. ON activity was followed by a pause before the visual stimulus was turned off. To determine whether the pause reflected the absence of excitatory drive or inhibitory conductance, we injected depolarizing currents juxtasomally, which enabled us to observe inhibition as decreased discharges. The pause was observed even under this condition, suggesting that inhibitory input caused the pause. We further found that local application of a mixture of gamma aminobutyric acid $(GABA)_A$ and $GABA_B$ receptor antagonists additively diminished the pause. These results indicate that GABAergic inputs produce transient ON responses by attenuating excitatory activity through the cooperative activation of $GABA_A$ and $GABA_B$ receptors, allowing sSC neurons to act as a saliency detector.

Keywords: $GABA_A$ receptor, $GABA_B$ receptor, juxtacellular recording, saliency, transient visual response, detection

Detecting an object that suddenly appears in the visual field is crucial for animals because their survival may depend on whether the object is beneficial or harmful. Transient, but not persistent, spike discharges in the sensory systems should encode the detection of the appearance. One of the candidate nuclei for this function is the superior colliculus (SC), a midbrain structure that is critical for visuomotor information processing (Sparks, 1986; Isa and Sparks, 2006). The superficial layer of the SC (sSC) receives visual inputs from the retina and the primary visual cortex (V1) in a retinotopically organized manner (Dräger and Hubel, 1976; May, 2006). sSC neurons exhibit transient spike discharges, called ON responses, immediately after the appearance of a visual stimulus (Schiller and Koerner, 1971; Berman and Cynader, 1972, 1975; Cynader and Berman, 1972; Goldberg and Wurtz, 1972; Dräger and Hubel, 1975; Rhoades and Chalupa, 1977; Marrocco and Li, 1977; Moors and Vendrik, 1979; Wang et al., 2010), implying that the sSC could detect the appearance of the object. Indeed, lesions or inactivation of the SC cause a detection deficit of visual stimuli (Butter et al., 1978; Overton and Dean, 1988; Fitzmaurice et al., 2003).

The sSC consists of several cell types, including excitatory and inhibitory neurons (Mize, 1992; Endo et al., 2003, 2005; May, 2006; Kaneda et al., 2008). Previous intracellular recording and labeling studies elucidated that some sSC neurons responded to moving or static visual stimuli with short bursts of spike discharges (Mooney et al., 1985, 1988). However, whether other cell types, specifically putative gamma aminobutyric acid

(GABA)ergic neurons, could respond in a similar manner is unclear. The mechanisms that make ON responses transient also remain largely unknown. A previous study demonstrated the different roles of $GABA_A$ and $GABA_B$ receptors on visual information processing, but did not precisely examine the effects of GABA receptor blockade on the nature of transient ON activity (Binns and Salt, 1997). Our previous study in slice preparations demonstrated that GABAergic sSC interneurons regulate the duration of discharges through a mechanism of feed-forward inhibition mediated by both $GABA_A$ and $GABA_B$ receptors in non-GABAergic and GABAergic sSC neurons (Kaneda et al., 2008), suggesting that GABAergic neurons contribute to transient activity. However, whether the same mechanism is at work *in vivo* has not been examined. Thus, the aims of the present study were to determine which cell types respond transiently to the appearance of a visual stimulus and what cellular and synaptic mechanisms underlie these responses. We used a juxtacellular recording/labeling technique (Pinault, 1996) that enabled us to identify cell morphology *post hoc* by loading neurobiotin into sSC neurons that had exhibited transient activity in response to a static visual stimulus in anesthetized mice. We also utilized a combined technique of single unit recording with local drug injection to determine which GABA receptor subtypes contribute to producing the transient responses. Our results demonstrate that most types of sSC neurons exhibit transient ON responses followed by a pause due to the cooperative activation of $GABA_A$ and $GABA_B$ receptors, and this pause shapes transient ON responses in the sSC.

EXPERIMENTAL PROCEDURES

The experimental protocols followed the guidelines of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and were approved by the Institutional Animal Care and Use Committee of National Institutes of Natural Sciences. All efforts were made to minimize the suffering and number of animals used in this study.

Visual stimulation and single-unit recordings

Male and female C57BL/6 mice aged from 8 to 15 weeks were anesthetized with urethane (1.2–1.5 g/kg in saline, i.p.), and the head was placed in a stereotaxic apparatus (Narishige, Tokyo, Japan). Dexamethasone (2.0 mg/kg) was injected subcutaneously. Additional urethane (0.2–0.3 g/kg) was administered as required. The incision was infiltrated with lidocaine (xylocaine jelly). The animal's body temperature was maintained at 37°C with a rectal thermoprobe connected to a heating pad (BWT-100, Bioresearch Center, Nagoya, Japan). Heart rate was continuously monitored throughout the experiment. The left eye was covered with silicone oil (FK-54, ShinEtsu, Tokyo, Japan). After performing a craniotomy to expose the cortex overlying the right SC, the exposed area was covered with agarose (2% in saline). A glass electrode filled with 2% neurobiotin (Vector Lab., Burlingame, CA, USA) in 0.5 M KCl (10–15 MΩ) was used for juxtacellular recording and labeling. A tungsten electrode (5–8

MΩ, FHC Inc., Bowdoin, ME, USA) attached with a silica capillary tube (40-µm inner diameter, 104-µm outer diameter; Polymicro Technologies Inc., Phoenix, AZ, USA) connected to a 1-µL Hamilton microsyringe was used for extracellular recording with local drug injections (modified from Kita et al., 2004; Kaneda et al., 2005; Tachibana et al., 2008). The distance between the tube orifice and the electrode tip was 250–300 µm. A micromanipulator was used to vertically lower electrodes into the brain at a distance of 0.6–0.8 mm lateral to the midline and 3.4–3.8 mm posterior to the bregma until visual responses to nearly the whole visual field stimuli were detected. Upon entering the sSC, the electrodes were slowly advanced, and single unit activities were recorded. Electrical signals were bandpass-filtered (0.3–6 kHz) and amplified with a Multiclamp 700B amplifier (1,000×, Molecular Devices) before being captured on a computer using an analog-to-digital card (National Instruments).

Visual stimuli were generated by MATLAB (Mathworks, Natick, MA, USA) programs using the Psychophysics Toolbox extensions (Brainard, 1997; Pelli, 1997; Kaneda et al., 2011, 2012). The stimuli were displayed on a 17-inch liquid crystal display monitor placed 25 cm from the mouse's left eye contralateral to the recorded (right) hemisphere. The right eye, which was ipsilateral to the recorded hemisphere, was covered. To determine the receptive field of sSC neurons, 6° light spots (55–60 cd/m^2), which induced the strongest responses in most sSC neurons under our experimental conditions (Kaneda et al., 2012), were flashed at

different locations on a gray background (6–8 cd/m^2), and the spike rate during the 1-s response to both flash onset and offset were calculated for each location. A custom-made program using MATLAB (Mathworks) generated a peristimulus time histogram (PSTH) with 10 ms time bins from all the responses to visual stimulation. The center of the receptive field was defined as the location where the maximum number of spikes per second was observed in the PSTH. After determining the center of the receptive field, the same light spot (6°, 55–60 cd/m^2) was presented for 300 ms. Each stimulus was repeated at least 4 times. An inter-stimulus interval of 5–7 s was introduced to minimize response adaptation. Spikes occurring in the first 30–330 ms after stimulus onset were binned as part of the ON responses of the cell, and subsequent spikes between 330 and 630 ms were binned into the OFF responses. In the majority of cells, a pause was observed between the ON and OFF responses. The peak firing rates of the ON responses were determined from the PSTHs. When neurons did not exhibit spontaneous firings, the duration of ON responses was measured between the first spike evoked immediately after visual stimulus and the last spike just before the pause. When neurons exhibited spontaneous firings, the onset of the ON responses was determined as the time of the first bin of the PSTHs where firing frequency exceeded the mean spontaneous firing rates, which were calculated by the number of spikes during pre-stimulus 300 ms, by $\geq$ 2SDs, and the end of the ON responses were defined as the time point of the last spike before the pause.

Spike induction by juxtasomal current injection

Juxtacellular recordings and extracellular single unit recordings in the sSC can address spike discharges that reflect spontaneous activity and/or excitatory inputs induced by sensory stimulus. However, investigating subthreshold inhibitory responses with these techniques is nearly impossible. To determine whether inhibitory inputs play a role in inducing the transient ON responses and pauses in the sSC, spike discharges were elicited by injecting depolarizing current pulses juxtasomally to originally silent neurons. After confirming stable ON, pause, and OFF responses, we injected a depolarizing current for 1.4 s into the recorded cells and adjusted the amount of current (1–5 nA), which resulted in inducing 14–25 spikes for each 1.4-s injection without visual stimulation. Then, the visual stimulus (300 ms) was re-applied 400 ms after the onset of the current injection (Fig. 3A). The duration of 1.4 s for current injection was selected to monitor stable firings before (400 ms) and after (400 ms) visual stimulus and to examine the effect of current injection on visual responses that were usually observed for about 600 ms. 10–20 trails were used to produce PSTHs. Under these conditions, we could detect an inhibition as a reduction or pause in spiking activities.

Juxtacellular labeling and histology

After the recordings, neurobiotin was injected with positive current pulses (3–7 nA, 1-s

duration, 50% duty cycle) delivered through the amplifier for 5–12 min while observing the discharge pattern of the injected neuron. The mice were sacrificed 0.5–1 hr after juxtacellular labeling with an overdose of sodium pentobarbital (80 mg/kg, i.p.), perfused transcardially with 0.05 M phosphate-buffered saline (PBS, pH 7.4) and fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed, postfixed for 1–2 d, cryoprotected in 30% sucrose in 0.1 M PB (pH 7.4), and sectioned (100-μm thickness). To visualize neurobiotin-labeled neurons, sections were washed in PBS, incubated with 0.6% H_2O_2 in methanol for 30 min to reduce endogenous peroxidase activity, and washed in PBS. Then, they were incubated with an avidin-biotin-peroxidase complex (ABC Elite kit; Vector Laboratories, Burlingame, CA, USA) for 2.5 hr at room temperature. After washing, the sections were reacted with 3,3′-diaminobenzidine (DAB) solution (0.01% DAB, 1% nickel ammonium sulfate, 0.0003% H_2O_2 in 0.01 mM Tris-buffered saline) for 30 min. The sections were rinsed in Tris-buffered saline and PBS. They were mounted on gelatin-coated slides, dehydrated, and cover slipped. The labeled cells were photographed with digital microscopes (Axioplan2, Zeiss, Oberkochen, Germany and BIOREVO, BZ-9000, Keyence, Osaka, Japan). The soma and dendrites of neurobiotin-labeled neurons were reconstructed with a camera lucida attached to a light microscope (BX51, Olympus, Tokyo, Japan).

Local drug injection during unit recordings

To investigate the contribution of $GABA_A$ and $GABA_B$ receptors to visual stimulus-induced responses, the effects of the $GABA_A$ receptor antagonist gabazine (1 mM; Sigma-Aldrich, St Louis, MO, USA), the $GABA_B$ receptor antagonist CGP52432 (CGP, 1 mM; Tocris), or a mixture of gabazine (1 mM) and CGP (1 mM) were examined during unit recordings. Both drugs were dissolved in 0.1 M PBS. After consistent and stable visual responses to at least 15 repeated stimuli, a total volume of 0.06–0.2 μL of each drug solution or vehicle (0.1 M PBS, 0.2 μL) was locally injected in the vicinity of recorded neurons at a rate of 0.04 μL/min using a micropump (ESP-32, EICOM, Kyoto, Japan) connected to a Hamilton microsyringe and a silica tube as described above. Drug doses were selected to be in the range of those used in previous studies using local administration of these compounds *in vivo*, where the antagonists effectively and selectively antagonized GABA receptor-mediated responses (Galvan et al., 2005; Kita et al., 2004, 2006; Tachibana et al., 2008, 2011). Because the effects of drugs appeared within 5 min in all cases, we presented PSTHs created just before and 5 min after the drug injections from 10-20 trials.

Data analysis

Data were analyzed with MATLAB (Mathworks). All values are expressed as mean ± SEM. Differences between groups were assessed with two-tailed paired Student's *t*-test and were

considered statistically significant if $P < 0.05$.

RESULTS

The majority of sSC neurons exhibit ON + Pause + OFF responses

The majority of sSC neurons reportedly exhibit both ON and OFF responses in response to a static visual stimulus (Schiller and Koerner, 1971; Berman and Cynader, 1972, 1975; Cynader and Berman, 1972; Goldberg and Wurtz, 1972; Dräger and Hubel, 1975; Marrocco and Li, 1977; Rhoades and Chalupa, 1977; Moors and Vendrik, 1979; Wang et al., 2010). However, among several types of sSC neurons (Mize, 1992; May, 2006), which type showed the ON and OFF responses was not necessarily clear. Thus, we firstly investigated morphologies of sSC neurons that exhibited the ON and OFF responses to static visual stimuli using a juxtacellular recording/labeling technique, which enabled us to study the morphology of recorded neurons (Pinault, 1996). When a 6° visual stimulus was presented on the display, almost all sSC neurons exhibited transient spike discharges immediately after (~50 ms) visual stimulus onset, which were determined as ON responses. These were followed by another transient spike discharges immediately after visual stimulus offset, which were determined as OFF responses. Figure 1A shows example traces to the visual stimulus. Because of the juxtacellular configuration, spikes exhibited a positive-negative sequence (Fig. 1A, inset). PSTHs calculated from spike numbers of the cell shown in Fig. 1A

revealed that this cell exhibited clear ON responses that were followed by a pause and subsequent OFF responses (hereafter referred to as ON + Pause + OFF responses; Fig. 1B). We also found other 2 types of responses: the first consisted of an ON response followed by a pause (hereafter referred to as ON + Pause responses; Fig. 1C) and the second type demonstrated only OFF responses (Fig. 1D). We found that 70 (88.6%), 5 (6.3%), and 4 (5.1%) out of 79 neurons showed ON + Pause + OFF, ON + Pause, and OFF responses, respectively (Fig. 1E), indicating that the majority of sSC neurons show ON + Pause + OFF responses. Among these neurons, some showed spontaneous firings (27 of 70 ON + Pause + OFF, 3 of 5 ON + Pause, and 2 of 4 OFF response neurons; Fig. 1E). There was no statistical difference in the ratio of spontaneously firing neurons among three groups (P = 0.884, Chi-square test).

To investigate the morphologies of sSC neurons that exhibited the transient ON responses, we injected depolarizing currents juxtasomally to introduce neurobiotin into 39 out of the 70 neurons that exhibited the ON + Pause + OFF responses and successfully identified the morphologies of 18 neurons (Fig. 2A). We could not recover or had to discard morphological data for the remaining 21 neurons because labeling was not observed in 6 cases, it was too faint to trace dendritic arbors in 5 cases, or 2 or more neurons were labeled in 10 cases (although only 1 neuron was targeted in each of those recordings). Based on the location of soma and somatodendritic morphologies (Langer and Lund, 1974; Tokunaga and

Otani, 1976; Labriola and Laemle, 1977; Mooney et al., 1985, 1988; Edwards et al., 2002), we classified the labeled neurons into 7 types (Fig. 2A), including wide-field vertical (WFV; n = 4; Fig. 2B), narrow-field vertical (NFV; n = 2; Fig. 2C), piriform (n = 4; Fig. 2D), multipolar (n = 3; Fig. 2E), marginal (n = 3; Fig. 2F), stellate (n = 1; Fig. 2G), and horizontal (n = 1; Fig. 2H). Thus, almost all sSC neuronal types exhibited ON + Pause + OFF responses.

We divided the 18 neurons into 9 putative excitatory (4 WFV, 2 NFV, and 3 marginal cells) and 9 putative inhibitory neurons (4 piriform, 3 multipolar, 1 stellate, and 1 horizontal cells) to ask whether any differences in the ON response and the following pause could exist between groups. However, parameters such as ON response latency (excitatory, 44.8 ± 4.2 ms vs. inhibitory, 51.2 ± 5.5 ms; P = 0.367), ON response duration (excitatory, 94.4 ± 16.5 ms vs. inhibitory, 86.9 ± 16.7 ms; P = 0.754), peak firing rate (excitatory, 150.1 ± 26.6 Hz vs. inhibitory, 128.4 ± 22.3 Hz; P = 0.540), pause duration (excitatory, 230.3 ± 14.8 ms vs. inhibitory, 255.2 ± 10.7 ms; P = 0.192), and OFF response latency that was defined as the period between stimulus removal and the initiation of the OFF response (excitatory, 69.4 ± 6.3 ms vs. inhibitory, 93.2 ± 12.3 ms; P = 0.111) exhibited no significant difference between the groups. On the other hand, the latency of ON response was shorter than that of OFF response in both excitatory (ON latency, 44.8 ± 4.2 ms vs. OFF latency, 69.4 ± 6.3 ms; P = 0.005) and inhibitory neurons (ON latency, 51.2 ± 5.5 ms vs. OFF latency, 93.2 ± 12.3 ms; P = 0.009).

Inhibitory inputs generate the pause

We next examined the synaptic mechanisms underlying pause generation that may contribute to shaping transient ON responses. We hypothesized that the pause might be mediated by 1) the absence of excitatory inputs, 2) inhibitory inputs, 3) depolarization block of sodium channels, or 4) activation of K^+ channels following spikes. To distinguish these possibilities, we juxtasomally injected depolarizing currents into recorded cells and elicited spikes before, during, and after visual stimulus presentation. If the pause was due to the absence of excitatory inputs, it would be expected that the current-induced spikes would not be diminished by visual stimulation. If it was mediated by inhibitory inputs, it would be anticipated that the current-induced spikes are reduced or ceased by visual stimulus. Finally, if the pause was due to depolarization block of sodium channels or activation of K^+ channels, it would be predicted that ON response duration should be shortened because depolarizing current injection would accelerate the depolarization block of sodium channels or current injection-induced spikes should enhance K^+ channel activation, resulting in prolonged duration of the pause following the ON responses. In this experiment, we chose sSC neurons that did not fire spontaneously and showed a clear pause between the ON and OFF responses (Fig. 3A1). The results shown in Fig. 3A strongly support the second possibility, because the pause was still observed when the visual stimulus was applied (Fig. 3A2, arrow). Moreover,

its duration was not prolonged, rather it tended to be reduced under this condition (before current injection, 211.0 ± 55.1 ms vs. during current injection, 174.0 ± 64.0 ms, n = 5, P = 0.086, paired Student's t-test). Additionally, some sSC neurons, including morphologically identified 8 neurons, exhibited spontaneous firings before visual stimulus presentation, and these firings ceased immediately after the ON response (a clear pause was observed between ON and OFF responses; Fig. 3B). These results strongly support the possibility that inhibitory inputs contribute to generating the pause in visual responses of sSC neurons.

$GABA_A$ and $GABA_B$ receptors cooperatively generate the pause

We next examined which types of GABA receptors mediate the inhibitory inputs that mediate the pause by performing extracellular single unit recordings combined with a local drug injection technique. A tungsten electrode was attached to silica tubing that was connected to the microsyringe, and either the $GABA_A$ antagonist gabazine (1 mM), the $GABA_B$ antagonist CGP (1 mM), or the mixture of both antagonists was injected locally to the recorded neurons that exhibited clear ON + Pause + OFF responses. We first confirmed that vehicle (0.1 M PBS) injection did not affect either the peak firing rate or the duration of ON responses (peak firing rate, predrug, 122.0 ± 21.5 Hz vs. vehicle, 117.2 ± 18.9 Hz, n = 6; P = 0.509, paired Student's t-test; ON duration, predrug, 63.3 ± 9.6 ms vs. vehicle, 65.0 ± 9.7 ms, n = 6; P = 0.809 Fig. 4A). Local application of gabazine significantly increased the peak firing rate of

the ON responses (predrug, 142.6 ± 30.3 Hz vs. gabazine, 281.8 ± 62.7 Hz, $n = 5$; $P = 0.042$, paired Student's t-test; Fig. 4B1–3). However, the duration of ON responses was not significantly affected (predrug, 56.0 ± 5.1 ms vs. gabazine, 56.0 ± 8.1 ms, $n = 5$; $P = 0.098$; Fig. 4B1, 2, 4). On the other hand, while CGP injection did not affect the peak firing rate (predrug, 115.4 ± 19.2 Hz vs. CGP, 143.4 ± 25.9 Hz, $n = 5$; $P = 0.124$; Fig. 4C1–3), the duration of ON responses was slightly but significantly prolonged (predrug, 77.0 ± 5.8 ms vs. postdrug, 92.0 ± 3.7 ms, $n = 5$; $P = 0.028$; Fig. 4C1, 2, 4). Finally, the injection of the mixture of gabazine and CGP remarkably augmented the peak firing rate of the ON responses (predrugs, 141.9 ± 21.2 Hz vs. gabazine + CGP, 298.6 ± 21.5 Hz, $n = 5$; $P = 0.0003$; Fig. 4D1–3) and significantly prolonged their duration (predrugs, 68.0 ± 8.6 ms vs. gabazine + CGP, 230.0 ± 15.2 ms, $n = 5$; $P = 0.0021$; Fig. 4D1, 2, 4), leading to a shortened pause. The effects of drugs usually continued over 40 min. We found the recovery from drug effects in 1 gabazine-treated, 2 CGP-treated, and 1 gabazien + CGP-treated neurons. Thus, these results indicate that $GABA_A$ and $GABA_B$ receptors cooperatively regulate the ON and Pause responses.

DISCUSSION

In the present study, we have provided evidence that a variety of morphological types of sSC neurons exhibit ON + Pause + OFF responses and that $GABA_A$ and $GABA_B$

receptor-mediated inhibitions are likely responsible for generating the pause that is critical in shaping the transient ON responses.

We observed 3 types of visual responses, including ON + Pause + OFF, ON + Pause, and OFF responses, and the majority of sSC neurons exhibited the first type of response. Previous studies reported responses similar to the ON + Pause + OFF pattern in rodents (Dräger and Hubel, 1975; Rhoades and Chalupa, 1977; Wang et al., 2010), cats (Berman and Cynader, 1972, 1975), and monkeys (Schiller and Koerner, 1971; Cynader and Berman, 1972; Goldberg and Wurtz, 1972; Marrocco and Li, 1977; Moors and Vendrik, 1979), although they did not explicitly describe the pause between the ON and OFF responses. With the use of juxtacellular labeling, we revealed that the ON + Pause + OFF responses were observed in a variety of morphological types of sSC neurons. These include WFV and NFV cells, which are reported to be excitatory neurons (Isa et al., 1998; Saito and Isa, 2005; Kaneda et al., 2008), and stellate, piriform, multipolar, and horizontal cells that are considered to be inhibitory GABAergic neurons (Mize, 1992; Endo et al., 2003; Kaneda et al., 2008). Three marginal cells, which are projection neurons (Mooney et al., 1988) and have not been characterized as GABAergic (Mize, 1992), also exhibited the ON + Pause + OFF responses. These findings are of particular importance because both excitatory and inhibitory neurons exhibited the same response pattern, although their functions must be different from each other: as

excitatory sSC neurons are projection neurons (Mooney et al., 1988; Isa et al., 1998; Özen et al., 2000; Kaneda et al., 2008), they send visual information to nuclei outside the sSC. On the other hand, given that the majority of GABAergic neurons are interneurons with some exceptions (Mooney et al., 1988), their primary role is most likely to inhibit excitatory and inhibitory neurons within the sSC.

The sSC contains silent neurons that fire only when a visual stimulus is presented (Dräger and Hubel, 1975; Marrocco and Li, 1977; Rhoades and Chalupa, 1977; Wang et al., 2010). One of the methods used to detect inhibitory inputs in these neurons is direct measurement of hyperpolarizing currents or membrane potential changes with whole-cell or intracellular recordings (Tao and Poo, 2005; Lien et al., 2006). However, if neurons exhibit spontaneous firing, inhibitory inputs can be detected as a reduction or cessation of firing. We took advantage of juxtacellular recording and injected a depolarizing current to induce spikes that allowed us to detect inhibitory inputs, which may be driven by visual stimuli. Under this condition, if the pause was a result of the absence of excitation, then spikes induced by the current injection could be observed between the ON and OFF responses. On the other hand, if the pause was a consequence of depolarization block of sodium channels or activation of K^+ channels, it would be prolonged due to the facilitation of depolarization block or increased activity of K^+ channels. However, we did not observe evidence for either of these scenarios.

Accordingly, we concluded that the pause between the ON and OFF responses was not due to the absence of excitatory inputs, a depolarization block, or activation of K^+ channels, rather it was produced by active inhibitory conductance (Fig. 3A). This was further supported by the results in spontaneously active neurons demonstrating firing cessation between the ON and OFF responses in response to the visual stimulus (Fig. 3B).

We found that some neurons in each response pattern exhibited spontaneous firings and there was no statistically significant difference in the ratio of spontaneously active neurons among groups, probably due to the paucity of neurons particularly in the ON + Pause and the OFF response groups. Additionally, the morphologically identified neurons also included spontaneous firing-positive neurons. Thus, it is difficult to classify neurons on the basis of their spontaneous firings at present. Further study to identify morphologies of neurons that show ON only and OFF only patterns would be necessary.

We demonstrated that both $GABA_A$ and $GABA_B$ receptors likely contribute to generating transient ON responses by producing the subsequent pause. Figure 5 schematically summarizes the possible interpretation of GABA antagonism on sSC neuronal activity.

We found that local gabazine application increased the peak firing rate but did not affect ON response duration. This finding was surprising because $GABA_A$ receptor blockade

significantly prolonged the duration of excitatory responses in other brain regions *in vivo* (Peng et al., 1996; Kita et al., 2006). The lack of effect for ON response duration may be accounted for by a series of related mechanisms. When $GABA_A$ receptors were blocked by local gabazine application, excitability of both non-GABAergic and GABAergic neurons in the sSC is enhanced, as we previously demonstrated *in vitro* (Kaneda et al., 2008). This subsequently increases glutamate and GABA release. The former activates glutamate receptors, including alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors, both of which are known to be essential for producing visual responses (Binns and Salt, 1994, 1996, 1998), and the latter activates densely expressed $GABA_B$ receptors, which have been reported to be expressed in both pre- and postsynaptic sites in excitatory and inhibitory sSC neurons (Binns and Salt, 2000; Endo and Isa, 2002; Kaneda et al., 2008). Thus, enhanced glutamate receptor activation increased the peak firing rates (Fig. 5B). Although $GABA_B$ receptor-mediated inhibition was augmented at the same time, the slower kinetics of $GABA_B$ responses may not significantly affect the peak firing rate, but it should effectively attenuate excitatory responses, with the net result being unchanged ON response duration (Fig. 5B).

On the other hand, CGP-mediated blockade of $GABA_B$ receptors may not affect the peak firing rate of ON responses because of slow postsynaptic $GABA_B$ inhibition, but their decay component would be prolonged due to relief from $GABA_B$ inhibition (Fig. 5C). At the same

time, both glutamate receptor-mediated excitation and $GABA_A$ receptor-mediated inhibition may be slightly enhanced by blocking presynaptic $GABA_B$ receptors (Endo and Isa, 2002; Kaneda et al., 2008). These balanced changes might also contribute to the unchanged peak firing rate of the ON responses.

Finally, when both $GABA_A$ and $GABA_B$ receptors were blocked, sSC neuronal excitation was significantly enhanced, which resulted in the prolonged duration and potentiated peak firing rates of the ON responses (Fig. 5D). However, it should be noted that although both $GABA_A$ and $GABA_B$ receptors contribute to shortening ON responses, it is difficult to distinguish whether prolonged ON responses are mediated by continuous drive from the retina and the V1, which might have been originally suppressed by GABAergic inhibition, or by long-lasting burst firings of sSC neurons, which were produced by intrinsic excitatory mechanisms in the sSC local circuit (Kaneda et al., 2008).

Although the combined application of $GABA_A$ and $GABA_B$ receptor antagonists significantly reduced the pause duration, they did not completely eliminate it (under our experimental conditions, the maximal duration of ON responses was, by definition, 300 ms. However, the duration of the ON responses after gabazine + CGP application was 230.0 ± 15.2 ms, Fig. 4D4). This suggests that other mechanism(s) might contribute to generating the pause. One possibility for this incomplete pause elimination may be derived from transient responses of retinal ganglion cells to a visual stimulus (Cleland et al., 1971; Levick, 1975).

We provided the evidence that the pause was caused by active inhibitory conductances by showing that the pause was still observed during the juxtasomal current injection in silent neurons or spontaneously active neurons (Fig. 3). However, because we did not test the effects of juxtasomal current injection on these pharmacologically manipulated neurons, we cannot exclude the possibility that the pause in these neurons might be attributable to a pause of retinal ganglion cells. An alternative candidate might be $GABA_C$ receptor-mediated inhibition given that $GABA_C$ receptors are reportedly expressed in the sSC (Schmidt et al., 2001; Boller and Schmidt, 2003; Kirischuk et al., 2003). However, unlike in the retina (McCall et al., 2002), synaptic currents through $GABA_C$ receptors were rarely observed in the sSC under normal conditions (Boller and Schmidt, 2003). Additionally, activation of $GABA_C$ receptors in the sSC results in disinhibition (Schmidt et al., 2001), suggesting that $GABA_C$ receptor blockade might reduce ON activity and augment the pause. Thus, although we did not examine the effect of $GABA_C$ receptor blockade, it could be speculated that the contribution of $GABA_C$ receptors in the generation and modulation of the ON and pause responses might be minor compared to that of $GABA_A$ and $GABA_B$ receptors. Further study is necessary to identify other contributor(s) to pause generation.

In the present study, we focused on the mechanisms for generating the transient ON response. It can be predicted that the mechanisms for generating the OFF response may be

more complicated than those for ON response. OFF responses may be caused by excitatory inputs from the retina and the V1 and rebound excitation that might be induced after inhibition is relieved (Edwards et al., 2002). Additionally, initiation timing and strength of OFF response might be modulated by duration and strength of the pause. Therefore, more systematic and sophisticated methods would be necessary for investigating the mechanisms of generating the OFF response in future studies.

The findings that a large number of sSC neurons exhibit the ON + Pause + OFF response and that the response pattern was observed in a variety of morphological types, including excitatory and inhibitory neurons, in the sSC strongly suggest that similar synaptic mechanisms may underlie the response pattern in both non-GABAergic and GABAergic neurons. This could be supported by the fact that both types of sSC neurons receive glutamatergic inputs from the retina and the V1 (Boka et al., 2006), as well as GABAergic inputs from local interneurons (Endo et al., 2005; Kaneda et al., 2008). Our juxtacellular labelings showed that half of the labeled cells were putative excitatory neurons (4 WFV, 2 NFV, and 3 marginal cells out of 18 labeled neurons, Fig. 2A), suggesting that randomly sampled neurons may contain both excitatory and inhibitory sSC neurons. This is probably due to the high percentage of GABAergic neurons in the sSC (Okada, 1974). Thus, it is most likely that both excitatory and inhibitory neurons were included in the experiments of

juxtasomal current injections and of local drug injections, although we did not examine the morphology of recorded neurons in those studies. Taken together, our data imply that at least some types of excitatory and inhibitory sSC neurons share similar mechanisms for producing the transient ON responses via curtailing excitatory responses by both $GABA_A$ and $GABA_B$ receptor-mediated inhibitions. Although functional meaning that multiple cell types in the sSC share the same response pattern and the GABA-mediated mechanisms for producing the transient ON responses is unclear at present, it might be speculated that such an transient activity in both excitatory and inhibitory neurons prevents unnecessarily prolonged burst activities in the sSC local circuit, which might affect the burst firings in the deeper layer of the SC through the direct signal transmission from the superficial to the deeper layer of the SC (Lee et al., 1997; Isa et al., 1998; Kaneda et al., 2008).

ON responses induced by a static visual stimulus in retinal ganglion cells and V1 neurons include both transient and persistent discharges (Hubel and Wiesel, 1959; Cleland et al., 1971; Levick, 1975). On the other hand, the majority of sSC neurons exhibit transient ON responses (Schiller and Koerner, 1971; Dräger and Hubel, 1975; Marrocco and Li, 1977; Rhoades and Chalupa, 1977; Moors and Vendrik, 1979; Wang et al., 2010). Thus, although a subset of sSC neurons exhibit persistent activity under some circumstances (Zhang et al., 1999), the continuous visual information derived from the retina and the V1 may be

temporally processed through the GABAergic mechanism in the sSC for generating transient signals. This mechanism of information processing may endow the sSC with the ability to detect the appearance, but not the persistent presentation, of an object in the visual field. This would allow the sSC to function as a saliency detector, which might be modulated by cholinergic inputs (Endo et al., 2005) and would be crucial for animals. This may be supported by the fact that lesions or inactivation of the sSC elicit a detection deficit of visual stimuli (Butter et al., 1978; Overton and Dean, 1988; Fitzmaurice et al., 2003), and by recent physiological studies that were combined with computational modeling to demonstrate that the sSC is critical for detecting salient objects in normal (Boehnke et al., 2011; Marino et al., 2012) and V1-lesioned monkeys (Yoshida et al., 2012).

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Figure legends

Fig. 1. sSC neurons exhibit 3 response patterns to a static visual stimulus. (A) Example traces showing that clear ON and OFF responses were induced by visual stimulus presentation. Gray areas in this and the following panels indicate visual stimulus presentation (size 6°, 300 ms duration). The inset shows the expanded trace of a single spike with a positive-negative sequence due to close apposition of the recording electrode to the cell. Five traces are superimposed. (B) A peristimulus time histogram (PSTH) in response to 300-ms light spots calculated from the spike responses obtained from the sSC neuron shown in (A). Clear ON + Pause + OFF responses were observed in response to the visual stimulus. (C) An example PSTH showing ON + Pause responses evoked by the visual stimulus. (D) An example PSTH showing OFF responses evoked by visual stimuli. (E) Populations of 3 response patterns of sSC neurons to the visual stimulus. The numbers in parentheses represent the numbers of spontaneously active neurons.

Fig. 2. A variety of morphological types of sSC neurons exhibit the ON + Pause + OFF responses in response to the visual stimulus. (A) A summary of morphological analysis. The numbers on the right indicate the numbers of neurons. The numbers in parentheses represent the numbers of spontaneously active neurons. Neurobiotin was loaded into 39 sSC neurons, and 18 were successfully identified. A variety of morphological types of sSC neurons were included. (B)–(H) Example photomicrographs and reconstructed somatodendritic

morphologies of recorded neurons that exhibited ON + Pause + OFF responses: (B) wide-field vertical (WFV), (C) narrow-field vertical (NFV), (D) piriform, (E) multipolar, (F) marginal, (G) stellate, and (H) horizontal cells. The scale bar (100 μm) applies to all drawings.

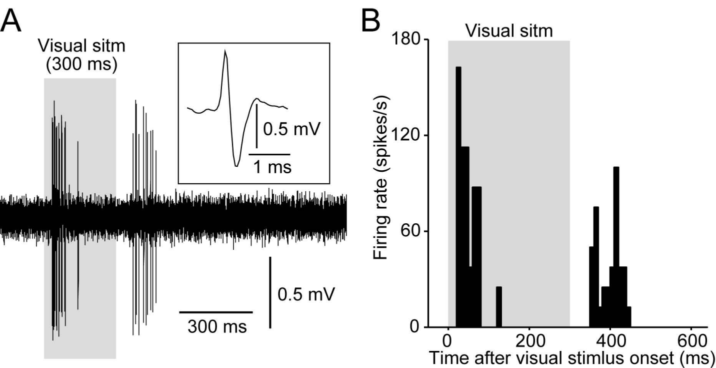
Fig. 3. The Pause is mediated by inhibitory inputs. (A1) Raster plots and a PSTH showing ON + Pause + OFF responses evoked by the visual stimulus without juxtasomal current injection in an sSC neuron that did not exhibit spontaneous firing. (A2) Raster plots and a PSTH obtained from the same neuron with juxtasomal depolarizing current injection from 400 ms before to 1000 ms after visual stimulus presentation onset. Even under this condition, the pause was still observed (arrow). Note that the uptick in firing rate in response to the current step onset includes artifacts (arrowhead). (B) Raster plots and a PSTH showing the ON + Pause + OFF responses evoked by visual stimulus without current injection in an sSC neuron that exhibited spontaneous firing.

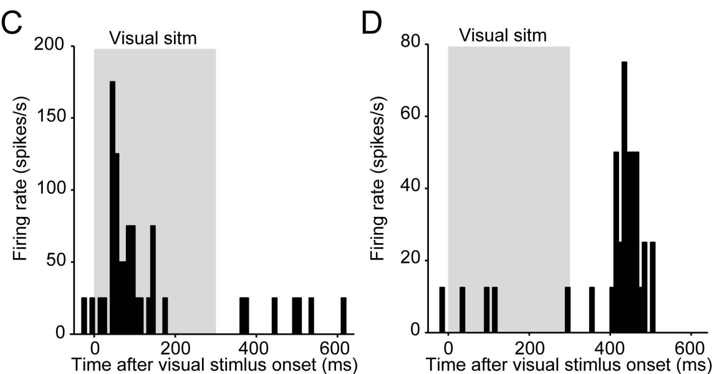
Fig. 4. $GABA_A$ and $GABA_B$ receptors cooperatively shape transient ON responses by inducing pauses. (A) Effects of local application of vehicle (0.1 M PBS) on visual responses in sSC neurons (n = 6). Neither peak firing rate nor ON duration was affected by PBS. (B) Effects of local application of the $GABA_A$ receptor antagonist gabazine (1 mM) on visual

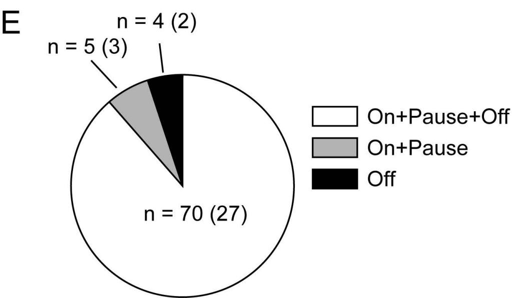
responses in sSC neurons ($n = 5$). Gabazine significantly increased the peak firing rate (B1, 2, 3) but did not affect ON response duration (B1, 2, 4). (C) Effects of local application of the $GABA_B$ receptor antagonist CGP52432 (CGP, 1 mM) on visual responses in sSC neurons ($n = 5$). CGP significantly prolonged ON response duration (C1, 2, 4) but did not affect the peak firing rate (C1, 2, 3). (D) Effects of local application of the mixture of gabazine (1 mM) and CGP (1 mM) on visual responses in sSC neurons ($n = 5$). The antagonist mixture significantly increased the peak firing rate (D1, 2, 3) and prolonged ON response duration (D1, 2, 4). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, paired Student's *t*-test.

Fig. 5. Schematic depicting the contributions of $GABA_A$ and $GABA_B$ receptor-mediated inhibitions to shaping transient ON responses. (A) In the control condition, visual stimuli induce both excitation (probably through AMPA and NMDA receptor activation) and $GABA_A$ and $GABA_B$ receptor-mediated inhibition. (B) When $GABA_A$ receptors are blocked, the excitabilities of both non-GABAergic and GABAergic neurons are enhanced, resulting in increased peak firing rates. However, because $GABA_B$-mediated slow inhibition is enhanced due to augmented activity of local GABAergic neurons, ON responses are not prolonged. (C) When $GABA_B$ receptors are blocked, the excitabilities of both non-GABAergic and GABAergic neurons are slightly increased due to the blockade of pre- and postsynaptic $GABA_B$ receptors. $GABA_A$-mediated fast inhibition competes with the excitation, leading to

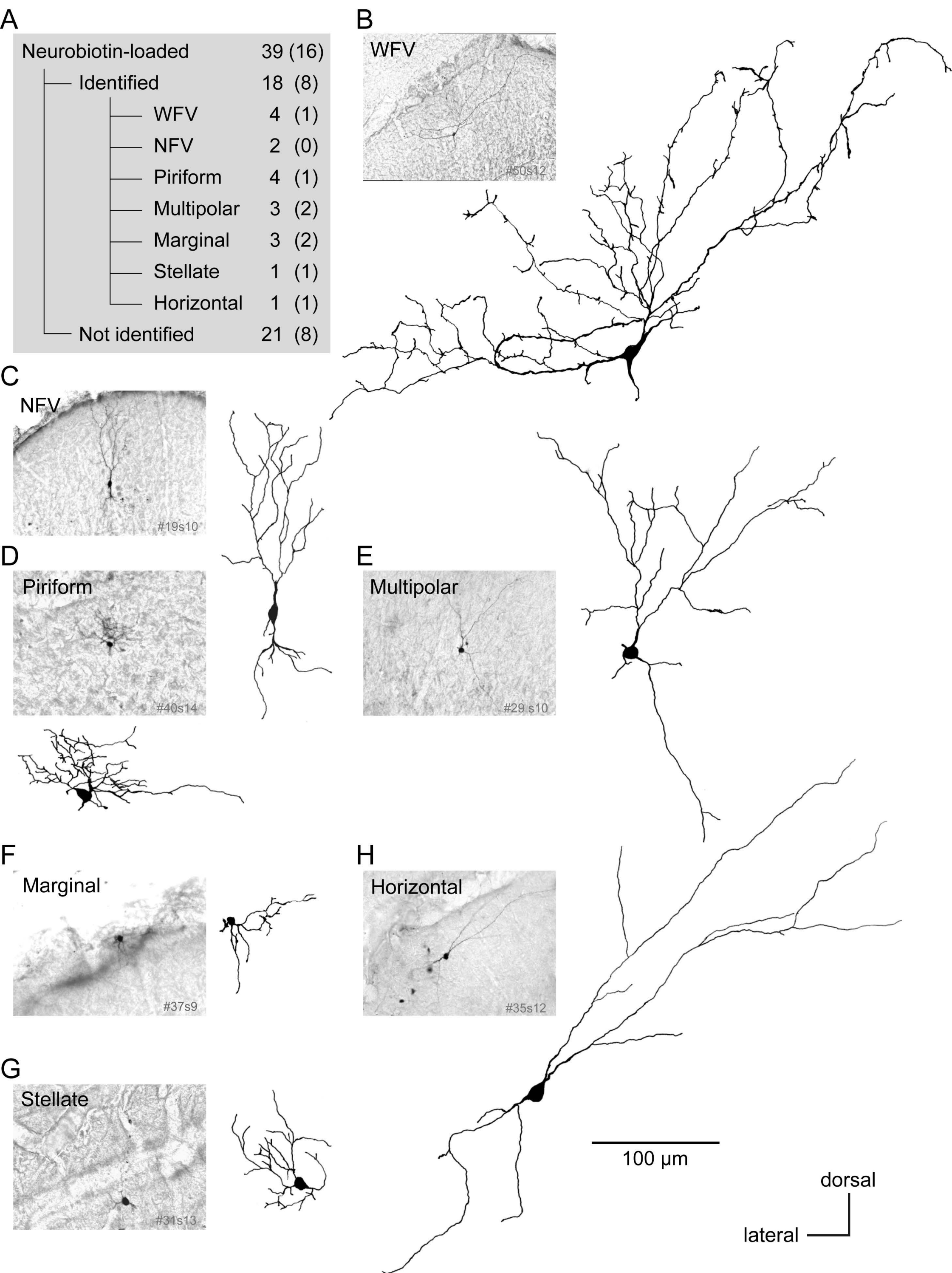
unchanged peak firing rates. However, ON response duration is prolonged due to relief from $GABA_B$-mediated slow inhibition. (D) When both $GABA_A$ and $GABA_B$ receptors are blocked, excitation is greatly enhanced, probably due to the long-lasting burst activity of excitatory neurons or persistent inputs from the retina and/or V1. The red, blue, and purple dashed lines in the upper panels in (B)–(D) indicate the magnitudes of excitation, $GABA_A$-mediated inhibition, and $GABA_B$-mediated inhibition in the control condition, respectively. The gray dashed lines in the lower panels in (B)–(D) indicate the magnitudes of ON response in the control condition.


A
Visual sitm
(300 ms)
0.5 mV
1 ms
0.5 mV
300 ms
B
Visual sitm
180
120
60
0
Firing rate (spikes/s)
0
200
400
600
Time after visual stimulus onset (ms)


C
Visual sitm
200
150
100
50
0
Firing rate (spikes/s)
0
200
400
600
Time after visual stimulus onset (ms)
D
Visual sitm
80
60
40
20
0
Firing rate (spikes/s)
0
200
400
600
Time after visual stimulus onset (ms)

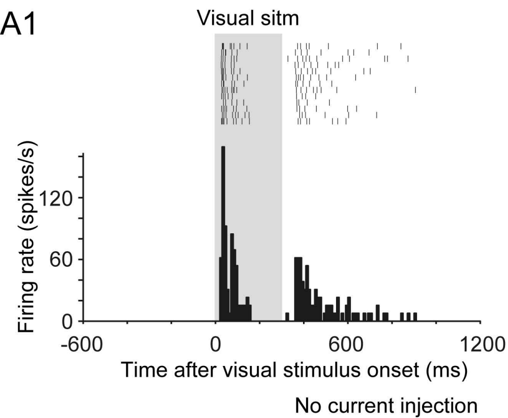

E
n = 4 (2)
n = 5 (3)
n = 70 (27)
On+Pause+Off
On+Pause
Off

Kaneda & Isa, Fig 2

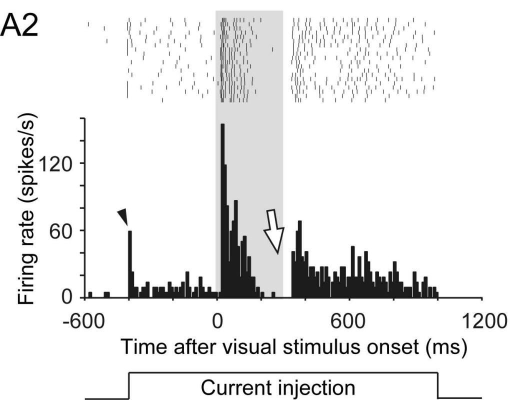


Kaneda & Isa, Fig 3

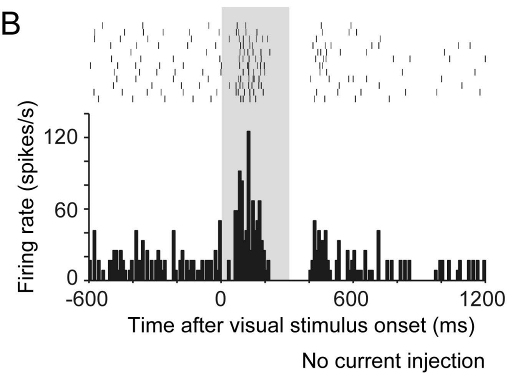
A1

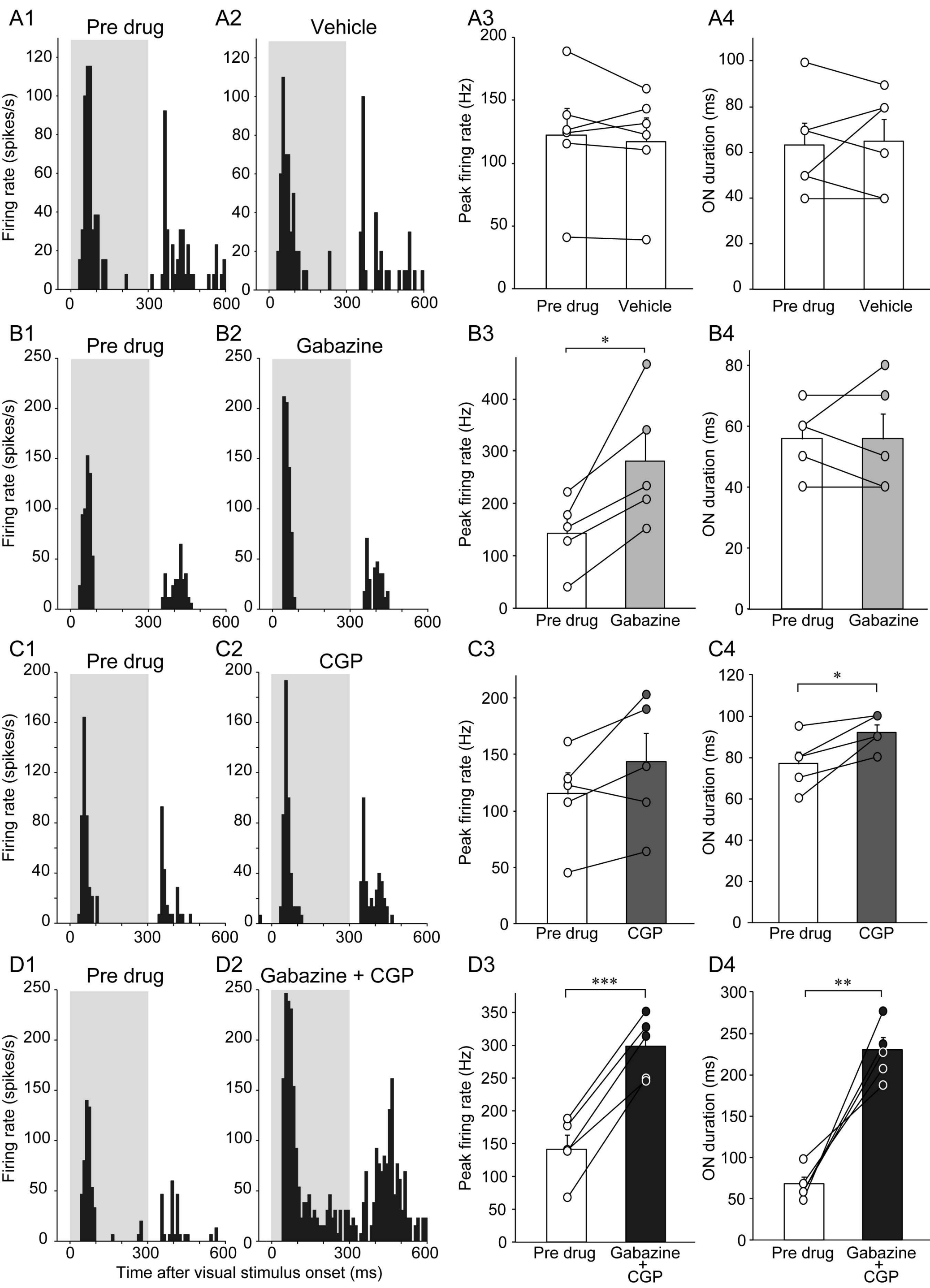


A2



B




A1
Pre drug
A2
Vehicle
A3
A4
Firing rate (spikes/s)
Peak firing rate (Hz)
ON duration (ms)
Pre drug
Vehicle
B1
Pre drug
B2
Gabazine
B3
*
B4
Pre drug
Gabazine
C1
Pre drug
C2
CGP
C3
C4
*
Pre drug
CGP
D1
Pre drug
D2
Gabazine + CGP
D3
***
D4
**
Pre drug
Gabazine + CGP
Time after visual stimulus onset (ms)

Kaneda & Isa, Fig 5