Article title: Laser-induced auditory perception: transtympanic laser irradiation of the cochlea from the outer ear evokes an auditory-related neural and behavioral response

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Abstract

The application of infrared laser stimulation to brain-machine interfaces, such as cochlear implants, has been discussed over the last decade because the infrared laser can activate spatially selected neural populations without delivering exogenous agents to tissues. Here, this study demonstrated for the first that the laser irradiation of the peripheral sensory organ, the cochlea, can induce an unequivocal behavioral auditory response. We proved that the animals, Mongolian gerbils (*Meriones unguiculatus*), can be classically conditioned with a laser stimulus in a similar way to an auditory stimulus. Additionally, the auditory masking significantly inhibited the laser-conditioned response, and the auditory-conditioned animals exhibited stimulus generalization to laser stimulation. In a subsequent experiment, simultaneous presentation of auditory and laser stimulation successfully elicited nonlinear amplification in both the auditory cortical and behavioral response. These results suggest that the infrared laser irradiation to the cochlea can evoke and possibly enhance auditory perception and has real potential to be implemented in an auditory prosthesis.

Introduction

Approximately 466 million people, 5% of the world's total population, suffer from hearing loss, causing severe communication difficulties and social isolation (*1, 2*). For the severely hearing impaired, implantable auditory devices including hybrid cochlear implants—using a hearing aid and cochlear implant technology in a single device—are one of the few options (*3*). However, the device requires surgical intervention, and hybrid implants in particular bear the risk of complications (*3-6*).

Since early research by Izzo, Richter, Jansen and Walsh Jr. (*7*) revealed that pulsed laser irradiation of the cochlea generates an electrophysiological cochlear response, the application of infrared laser stimulation to auditory prostheses has been discussed over the last decade. Potentially, the laser stimulation can activate spatially selective cell populations in a noncontact manner without exogenous agents to tissues (e.g., viral vector) (*8*). Some studies have investigated the detailed relationship between the irradiation site of the cochlea and laser-evoked neural response, indicating that laser could elicit spatially specific neural activities (*9, 10*). Our previous studies also leveraged its contactless feature and penetrability through thin tissues, and showed that transtympanic laser irradiation of the cochlea from the outer ear could elicit a cochlear response (*11-13*) and auditory cortical activities (*14*), bypassing the middle ear. These research indicated

that auditory prostheses with laser stimulation could provide more precise spectral information less invasively than conventional electric cochlear implants.

To date, little is known about laser-induced perceptual events, while several researchers have investigated the correlation between laser stimulation and physiological response at the peripheral (*7, 10, 15, 16*), midbrain (*17-20*), and cortical level (*14*). Matic, Robinson, Young, Badofsky, Rajguru, Stock and Richter (*21*) showed in cats that irradiating a cochlea with the laser increased the probability of turning their head to the irradiated side; however, because the animals response was their spontaneous reaction and was not associated with specific perception, the content of laser-evoked perception has not yet been identified.

For systematically evaluating the perceptual content we employed Mongolian gerbils (Fig. S1, S2), a standard animal model for auditory physiology with larger-sized cochleae and relatively high sensitivity to frequency range of human speech (e.g., *22, 23, 24*), and trained them to associate the reward, a drop of water (US: unconditioned stimulus), with either auditory, laser, or visual cues (CS: conditioned stimulus) using standard classical conditioning paradigm (see materials and methods for detail). Cochlear responses triggered by the laser and auditory stimulus during performing the conditioning task were recorded to correlate with behavioral responses. After evaluating laser-intensity

dependence of the behavioral response, we assessed the auditory masking effect on the laser-evoked behavioral response and tested stimulus generalization from auditory to laser stimulation. Subsequently, simultaneous presentation of auditory and laser stimulation was performed to evaluate the interaction and the possibility of enhancing auditory perception with simultaneous stimulation.

Results and discussion

Our data showed that subject animals were successfully trained with cochlear-laser stimulation as conditioned stimulus, and the amplitude of the laser-evoked compound action potential (CAP) was well corelated with the strength of conditioned response, the licking rate (Fig.1). Although some studies have attempted to show the possibility of manipulating the perception in terms of intensity dependency (*7, 25*) and spectral selectivity (*17, 18*) by comparing the auditory- and laser-evoked physiological response, no studies have revealed the relationship between the laser-evoked physiological response and behavioral consequences. Our data described that both laser-evoked CAP and behavioral responses increased depending on the radiant energy, and these dependences are comparable to those on the sound pressure level (Fig.1, A to F). The correspondence suggests that laser-evoked perception can be controlled by changing radiant energy

92 possibly in a similar way by the sound pressure level of auditory stimulation.

93 The laser-conditioned behavioral response was inhibited by the concurrent white
94 noises depending on the noise intensity, suggesting the laser stimulation of the cochlea
95 induced auditory perception (Fig.2). A previous research reviled that the laser-evoked
96 electrophysiological response was able to be masked by acoustic stimulation, indicating
97 the laser-evoked peripheral response was processed as auditory signals (*10*). No studies,
98 however, have directly evaluated behavioral consequences and provided compelling
99 evidence of the perception. Our study demonstrated that auditory stimulus systematically
100 masked both laser-evoked and auditory-evoked response, while not visually-evoked
101 behavioral response, licking behavior (Fig. 2, A to D, and Fig. S6). The reduction of
102 licking (delta licking rate: DLR) was well correlated with decrement of CAP amplitude
103 (difference of amplitude: diff amplitude) in both auditory- and laser-evoked response
104 group, suggesting that the disturbance of the laser-evoked neural responses by auditory
105 stimulus similarly affects perceptual events as the disturbance of the auditory-evoked
106 neural response. These results indicate that subjects processed the laser-evoked cochlear
107 response as a sound-induced signal at the perceptual level.

108 Studies of the stimulus generalization paradigm indicated that transtympanic laser
109 stimulation formed a similar perception as auditory stimulation (Fig.3). Stimulus

generalization in classical conditioning techniques has been widely used to examine the degree of the stimulus similarity in animal perception, and the generalization behavior is only observed when the unassociated stimulus is highly similar to the CS (*26, 27*). In this study, acoustically trained subjects showed stimulus generalization to transtympanic laser stimulation, which was never presented during the conditioning (Fig.3, A to H). This result suggests that laser-evoked perception was similar to auditory-evoked perception. Additionally, analysis of covariance (ANCOVA) showed that the laser-generated linear regression slope in the dependence of DLR on CAP in the acoustically trained subjects was significantly smaller than the auditory-generated linear regression slope (Fig.3I), while there is no difference between auditory- and laser-generated dependence in the subjects trained on each stimulus modality (Fig.1G). In other words, laser stimulation induced a lower behavioral response than auditory stimulation, although laser stimulation might elicit a similar perceptual level as auditory stimulation. Generally, CR amplitude peaked at the CS and gradually decreased as the similarity between the CS and the unassociated stimuli fell (*26, 27*). These results suggest that transtympanic laser stimulation elicited a similar but at least partially different perception as auditory stimulation. Laser-induced neural recording in the inferior colliculus (*17, 18*) and auditory cortex (*14*) have been carried out to estimate the relationships between the optical path

128 and spectral selectivity of the laser stimulation. Baumhoff, Kallweit and Kral (*20*)
129 indicated that laser-induced neural activation in the inferior colliculus (IC) is relatively
130 independent of the direction of the laser path and has a global activation pattern like IC
131 response elicited by clicking sound. However, Moreno, Rajguru, Matic, Yerram,
132 Robinson, Hwang, Stock and Richter (*17*) revealed that laser-evoked tonotopic response
133 in IC depends on the optical path, suggesting that the laser irradiation site of the cochlea
134 can characterize laser-evoked perception. Our results showing that laser-induced
135 perception is partially different from the perception elicited by the click train are more in
136 line with the Moreno, Rajguru, Matic, Yerram, Robinson, Hwang, Stock and Richter (*17*)
137 study than those by Baumhoff, Kallweit and Kral (*20*). Future studies using laser-trained
138 subjects transferring behavioral responses to several kinds of auditory stimuli (e.g.,
139 different frequency sounds) are required to assess a more complicated relationship
140 between optical path and laser-evoked perceptual content.

141 A simultaneous combination of auditory and laser stimulation with sub- or near-
142 threshold intensity elicits a greater neural and behavioral response than the auditory and
143 laser stimulus alone (Fig.4 and Fig. S7). Many related studies have focused on the
144 development of intracochlear stimulation systems to improve the spectral resolution of
145 cochlear implants using infrared laser stimulation (*21, 25, 28*) or optogenetic stimulation

146 (*29, 30*). However, so far, no study except for my previous research has investigated the
147 potential of infrared laser stimulation for reducing the invasiveness of surgical
148 implantation, which has the risk of damaging residual hearing (*31*), by leveraging the
149 contactless feature of laser stimulation (*11-14*). Reducing the risk of damaging residual
150 hearing caused by complications might improve speech comprehension by combing laser-
151 evoked perception with residual hearing more efficiently than the electro-acoustic
152 stimulation in hybrid cochlear implants. In this study, we examined the feature of the laser
153 interactions with the auditory evoked response by changing the relative timing of auditory
154 and laser stimulation. The data showed that the most vigorous auditory evoked response
155 was observed when both stimuli were presented synchronously (Fig. S9). In addition, the
156 simultaneous stimulation induced nonlinear facilitation of the neural and behavioral
157 response (Fig.4, A to D). These results suggest that synchronous presentation of auditory
158 and laser stimulation helps to improve hearing sensation.

159 Evaluating the safety margin of transtympanic laser stimulation is one of the
160 essential processes for the clinical application of laser auditory prostheses. Our previous
161 study demonstrated that a transtympanic laser under 6.6 W/cm^2 did not induce observable
162 thermal elevation and did not cause histological, electrophysiological, and behavioral
163 damage in Mongolian gerbils. This result suggests that a transtympanic laser of 6.6 W/cm^2

164 or weaker could be available without concern for acute injury (*13*). Our current data
165 showed that laser-evoked behavioral response started to be observed at approximately 1.2
166 mJ/cm^2 or more pulsed laser train at 4000 Hz, which was delivered for 500 ms (i.e.,
167 radiant energy of the stimulus was 2.4 W/cm^2), indicating that laser-evoked perception
168 could be generated in a safety margin of the laser stimulation (Fig.1). Additionally, we
169 recorded the temperature of laser irradiated cochlear surface, which was one of the main
170 reasons for laser-induced damage, to investigate the thermal build-up following laser
171 irradiation and found that observable thermal elevation did not occur until 5 s when the
172 laser stimulation with maximum radiant energy in the experiment (13.2 mJ/cm^2 at 4000
173 Hz) was presented (Fig. S8). This result indicates that short-time laser irradiation of
174 maximum radiant energy within 5 s does not generate dangerous thermal elevation for
175 eliciting perceptual events. Therefore, radiant energy to manipulate laser-induced
176 perception in this experiment is within the safety margin of the laser stimulation.
177 Although future safety evaluations of human transtympanic laser stimulation are required,
178 this study could show that controlling auditory perception without acute thermal damage
179 is feasible.

180 While our data is the first demonstration of auditory perceptual manipulation of
181 infrared laser stimulation alone or in combination with sound signals, it must be noted

182 that the experiment cannot rule out the possibility that laser-evoked neural and behavioral
183 responses were generated through the mediation of hair cell function. After the initial
184 study by Wells, Kao, Jansen, Konrad and Mahadevan-Jansen (*32*) demonstrated that laser
185 irradiation of sciatic nerves yielded CAP in *vivo*, some studies investigated the underlying
186 mechanism of laser-evoked neural response. These studies suggest that optical absorption
187 of water caused an instantiable temperature rise in cells (*33-35*), resulting in the reversible
188 alternation of the electric capacitance of the plasma membrane (*36*) or change in heat-
189 induced ion channel activity (*37*). The thermal build-up in cells of the cochlea also results
190 in basilar membrane vibration with lymph expansion causing hair cell depolarization (i.e.,
191 optoacoustic effect) (*20, 38, 39*) and direct activation of electrophysiological response in
192 hair cells (*40-42*); laser stimulation has the possibility of inducing hair cell depolarization.
193 To investigate the function of hair cells in laser-induced cochlear activation, some studies
194 (*20, 43, 44*) compared laser-evoked auditory responses in normal hearing and chemically
195 deteriorated cochlea, showing that laser-evoked cochlear responses were not observed in
196 the chemically impaired cochlea. These results suggest that the origin of the laser-evoked
197 cochlear response is hair cell-mediated.

198 In contrast, Richter, Bayon, Izzo, Otting, Suh, Goyal, Hotaling and Walsh Jr. (*45*)
199 reported that cochlear response induced by laser stimulation could be observed after 30–

200 40 dB chemical deafening of the auditory threshold. Tan, Jahan, Xu, Stock, Kwan,
201 Soriano, Xiao, Garcia-Anoveros, Fritzsch and Richter (*46*) demonstrated that laser
202 stimulation could induce CAP in the cochlea in congenitally hearing-impaired mice
203 whose cochlea lacks synaptic transmission between the inner hair cells and spiral
204 ganglion neurons. Additionally, the laser-induced response cannot be masked by white
205 noise when animals have a chemically deteriorated cochlea (*47*). These results suggest
206 that laser stimulation bypasses the need for hair cell depolarization and creates a cochlear
207 nerve response directly. This notion is partially in line with our previous findings (*12, 14*)
208 showing that the cochlear microphonics and CAP were recorded when the clicking sound
209 was presented, while pulsed laser irradiation of the cochlea induced only CAP as reported
210 in earlier studies (*7, 38*). In addition, our current result showed that laser stimulation
211 induced a partially different perception from the clicking sound, although the tonotopic
212 response in IC with laser stimulation might correspond to that with clicking sound in the
213 case that laser irradiation opoacoustically induced cochlear response (*20*). Future studies
214 are necessary for investigating the physiological mechanism of laser-evoked auditory
215 perception by comparing the effect of laser irradiation with different levels of peripheral
216 hearing impairment.

217 The clinical utility of laser stimulation depends on whether the laser directly

218 activates auditory nerves or whether the CAP was generated due to hair cells mediated
219 response. Generally, cochlear implants are reserved for profound hearing loss with few
220 functional hair cells (*3*); therefore, laser stimulation cannot be applied to cochlear
221 implants if the laser needs functional hair cells. From our current and previous data (*12*),
222 laser irradiation from the outer ear circumvented the function of the middle ear and
223 produced auditory perception, whereas it is still unclear whether the laser-evoked auditory
224 perception was elicited through the mediation of hair cells depolarization. This result
225 suggests that laser stimulation will only show clinical benefits as an alternative to
226 noninvasive middle ear implants. In other words, laser stimulation is unlikely to be
227 applied to cochlear implants without elucidating the detailed mechanism of laser-evoked
228 cochlear response. However, these days, hybrid cochlear implants, which combine a
229 cochlear implant with hearing aid devices in the same ear, are widely used among
230 moderate hard-of-hearing individuals who cannot comprehend speech conversation with
231 a hearing aid alone (*3*). In this study, simultaneous presentation of auditory and laser
232 stimulation generated a more massive neural and behavioral response than auditory and
233 laser stimulation alone (Fig.4). This result suggests that modest hearing-impaired patients
234 could receive significant hearing benefits from the simultaneous presentation of auditory
235 and laser stimulation compared to hearing aids alone. Although it is still unclear whether

transtympanic laser stimulation alone can be a possible alternative to electric stimulation in cochlear implants, it could help amplify hearing sensation by synchronously presenting auditory and laser stimulation like hybrid cochlear implants.

Together, this study revealed that transtympanic laser stimulation induced auditory perception in an intensity-dependent manner as auditory stimulation. A simultaneous combination of auditory and laser stimulation elicits greater auditory perception than the auditory and laser stimulus alone. These results suggest that transtympanic laser stimulation could help to improve hearing sensation in hearing loss patients whose hearing disability cannot be compensated for using a hearing aid alone. Our work provided the foundation for implementing transtympanic laser stimulation to auditory prostheses and will be an essential step for the clinical application of earphone-type noninvasive auditory prostheses with infrared laser-based technology.

Figure

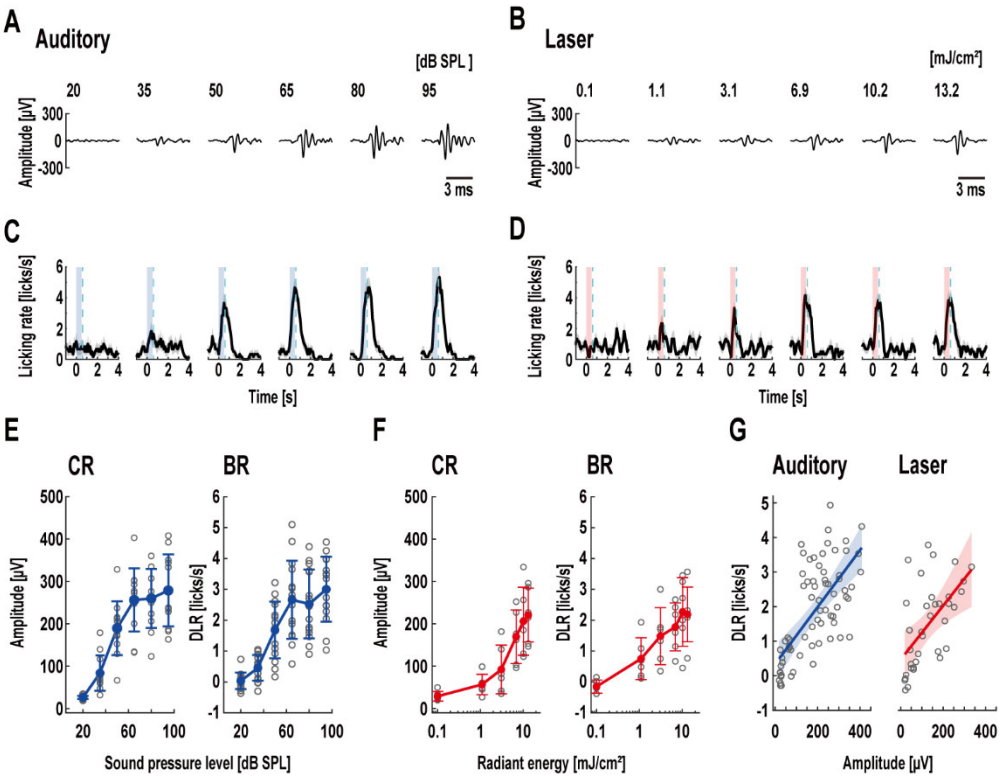

Fig.1 Intensity dependence of the cochlear response (CR) and the behavioral response (BR) elicited by auditory and laser stimulation. CAP from the auditory nerves with auditory (A) and laser (B) stimulation. Mean peristimulus time histogram (PSTH) of licking behavior elicited by auditory (C) and laser (D) stimulation. In (C) and (D), the blue and red zones indicate the auditory and laser stimulating periods. Gray areas show the standard error of the mean (SE) of each trial. Light blue dotted lines represent reward timing in a training session. Increasing the intensity of auditory and laser stimuli produced a systematic change in the CAPs and the mean PSTHs. The average amplitude of CAP and delta licking rate (DLR) with auditory (E) and laser (F) stimulation. Error

bars show standard deviation (SD). As the sound pressure level increased from 20 to 95 dB SPL, the average CAP amplitude and DLR increased from 26.14±3.87 to 278.63±84.83 (n=12; Mean±SD) and 0.03±0.27 to 3.00±1.05 (n=15), respectively. With increasing radiant energy from 0.1 to 13.2 mJ/cm^2, a steady increase of CAP amplitude and DLR was observed (CAP amplitude: from 28.92±11.34 to 220.71±63.38, n=6; DLR: from -0.15±0.21 to 2.19±0.89, n=6). (G) The correlation between CAP amplitude and DLR elicited by auditory and laser stimulation. Blue and red lines depict straight line fits, and blue and red areas show 95% confidence interval. Increasing CAP amplitude resulted in a systematic increment of DLR in auditory and laser stimulation conditions (auditory: r=0.80, ***P<0.001; laser: r=0.81, ***P<0.001; Pearson's correlation analysis). The slopes and y-axis intercepts of the fitted linear regression were not statistically different from each other (slope: F(1, 104)=0.046, P=0.832; intercepts: F(1,105)=0.093, P=0.761; ANCOVA). In (E to G), open gray circles show the individual data. Further intensity dependence of behavioral response is described in the supplemental figure (Fig. S5).

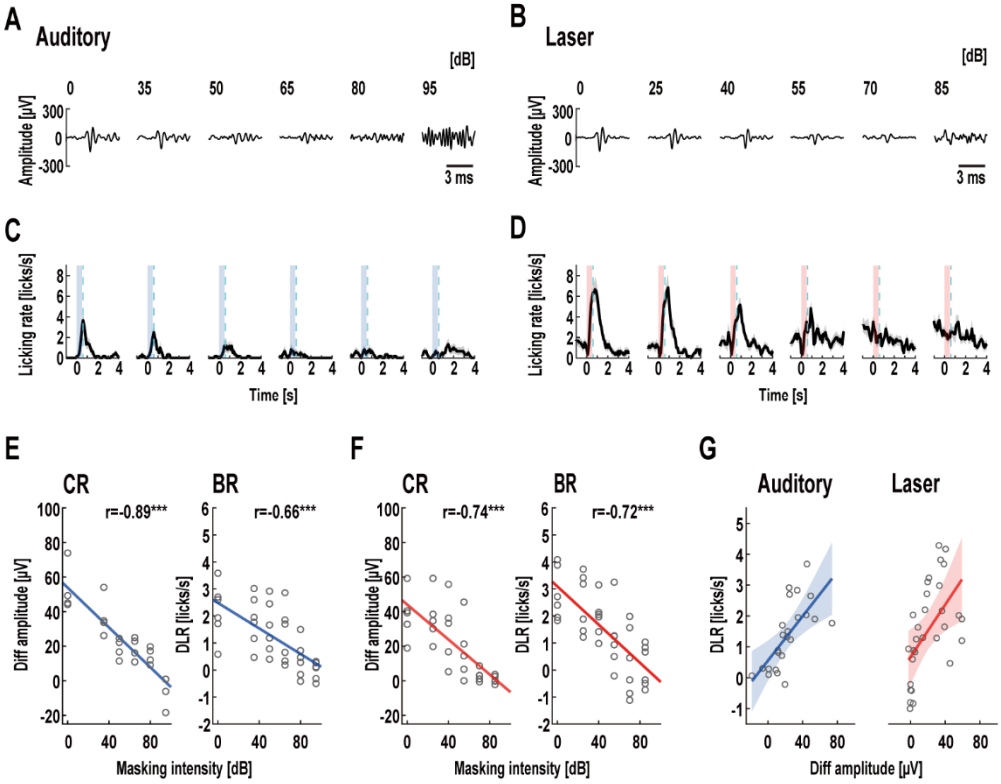

Fig.2 The auditory masking effect on CR and BR evoked by auditory and laser stimulation. The effect of auditory masking (0, 35, 50, 65, 80, 95 dB SPL) on CAP from the auditory nerves evoked by 80 dB SPL auditory (A) and 13.2 mJ/cm^2 laser (B) stimulation. Mean PSTH of licking behavior induced by 80 dB SPL auditory (C) and 13.2 mJ/cm^2 laser (D) stimulation. The blue and red zones in (C) and (D) indicate the auditory and laser-stimulating periods. Gray areas show SEMs of each trial. Light blue dotted lines represent expected reward time. Change in diff amplitude of CAP and DLR induced by auditory (E) and laser (F) stimulation with different masking intensities. As the intensity of the masker on auditory-evoked cochlear and behavioral response increased, CAP amplitude and DLR significantly decreased (CAP amplitude: r=-0.89, ***P<0.001; DLR:

287 r=-0.66, ***P<0.001; Pearson's correlation analysis). A similar tendency was observed
288 in laser-evoked CR and BR, where the intensifying sound pressure level of the masker
289 caused a significant decrease in CAP amplitude (r=-0.74, ***P<0.001; Pearson's
290 correlation analysis) and DLR (r=-0.68, ***P<0.001; Pearson's correlation analysis). (G)
291 Linear regression of DLR changes onto diff amplitude of CAP. Blue and red lines show
292 straight line fits, and blue and red areas show 95% confidence interval. A monotonous
293 increase in DLR was observed depending on the diff amplitude of CAP in auditory and
294 laser stimulation conditions (auditory: r=0.80, ***P<0.001; laser: r=0.81, ***P<0.001;
295 Pearson's correlation analysis). An ANCOVA showed no significant difference in slopes
296 (F(1,50)=0.177, P=0.676; ANCOVA) and y-axis intercepts (F(1,51)=0.741, P=0.393;
297 ANCOVA) of the auditory- and laser-induced relationship between diff amplitudes of the
298 peripheral neural response and DLR. In (E to G), open gray circles show the individual
299 data.
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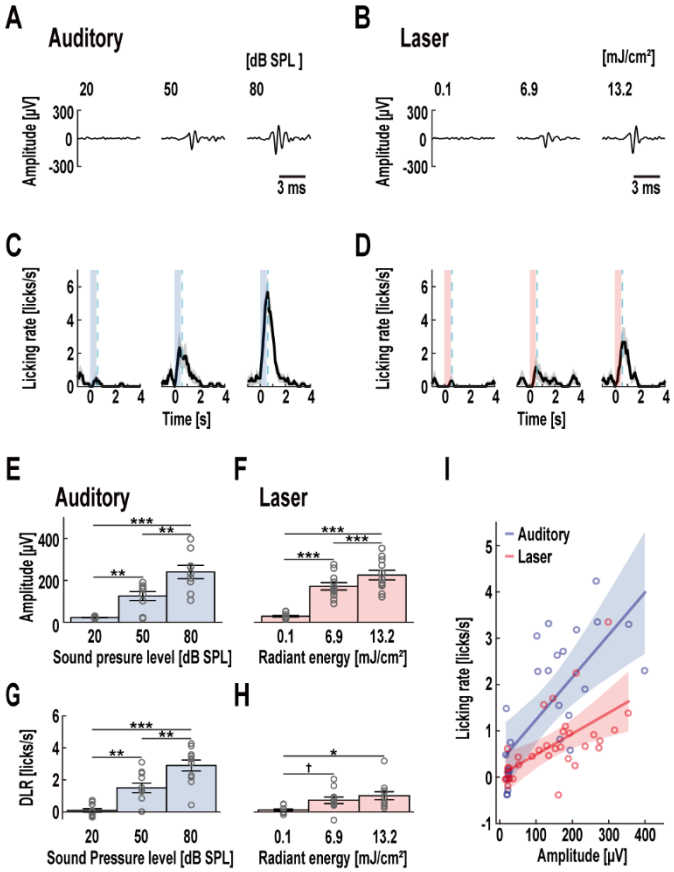

Fig.3 CR and BR in stimulus generalization paradigm from auditory stimulation to laser stimulation. CAP from the auditory nerves (A and B) and mean PSTH of licking behavior (C and D) elicited by auditory (20, 50, 80 dB SPL) and laser (0.1, 6.9, 13.2 mJ/cm^2) stimulation in the subjects trained on classical conditioning using auditory stimulation. The blue and red zones in (C) and (D) indicate the auditory and laser-stimulating periods. Gray areas show SEMs of each trial. Light blue dotted lines represent reward timing subjects expected. Mean CAP amplitude (E and F) and DLR (G and H) with auditory and laser stimulation. Error bars are described as SEMs. Open gray circles show the individual data. Auditory- and laser-induced CAP amplitude significantly

312 increased with stimulus intensity (auditory, 20 dB SPL: 22.45±1.37 μV, 50 dB SPL:
313 125.33±21.56 μV, 80 dB SPL: 240.79±31.71 μV, n=9; laser, 0.1 mJ/cm^2: 28.74±3.79 μV,
314 6.9 mJ/cm^2: 172.04±17.61 μV, 13.2 mJ/cm^2: 225.83±22.90 μV, n=11; Mean±SEM).
315 Laser stimulation induced licking behavior from the auditory-trained animals in an
316 intensity-dependent manner as auditory stimulation did. As the sound pressure level
317 increased from 20 to 80 dB SPL, DLR increased from 0.09±0.11 to 2.90±0.34 licks/s
318 (n=11). Laser-induced DLR gradually changed depending on radiant energy (0.1 mJ/cm^2:
319 0.12±0.07 licks/s, 6.9 mJ/cm^2: 0.73±0.20 licks/s, 13.2 mJ/cm^2: 1.01±0.25 licks/s, n=11).
320 In (E to H), there was a significant difference in response to each stimulus intensity, which
321 was compared by two-tailed paired *t*-test with Bonferroni correction for multiple
322 comparisons (†P<0.1, *P<0.05, ***P*<0.01, ****P*<0.001). (I) DLR as a function of CAP
323 amplitude induced by auditory and laser stimulation. Blue and red lines plus shaded areas
324 describe fitted linear regressions plus 95 % confidence intervals. Open blue and red
325 circles show the individual data. The correlation coefficients between CAP amplitude and
326 DLR were statistically significant in both auditory and laser stimulation conditions
327 (auditory: r=0.80, ****P*<0.001; laser: r=0.81, ****P*<0.001; Pearson's correlation
328 analysis). Comparing the linear regression model between auditory and laser stimulation
329 conditions revealed the difference of the slopes and the y-axis intercepts were statistically

330 significant (slopes: $F(1,56)=5.474$, $^{*}P<0.05$; y-axis intercepts: $F(1,57)=18.256$,

331 $^{***}P<0.001$; ANCOVA).

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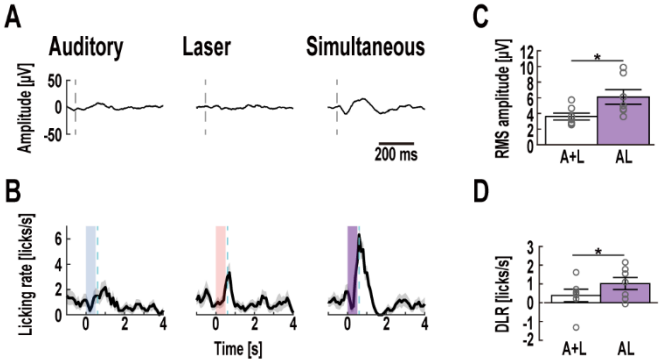

Fig.4 Facilitation of auditory evoked potential (AEP) and BR by the simultaneous presentation of auditory and laser stimulation with sub- or near-threshold intensity. Comparing AEP (A) and PSTH of licking behavior (B) elicited by auditory, laser, and simultaneous presentation of auditory and laser stimulation. In (A), gray dotted lines indicate stimulus onset. Blue dotted lines in (B) show the expected reward timing. Gray areas show SEMs of each trial. The blue, red, and purple zones indicate the auditory, laser, and simultaneous stimulating periods. Comparing the response elicited by auditory and laser stimulation alone with that by simultaneous stimulation described that a simultaneous combination elicited greater auditory cortical and behavioral response than auditory and laser stimulation alone. Response amplitude of linear summation of auditory- and laser-evoked response and simultaneous stimulation in AEPs (C) and mean PSTHs of licking behaviors (D). The amplitude evoked by simultaneous presentation in both neural and behavioral response was significantly higher than the amplitude of linearly summating response evoked by auditory and laser stimulation alone (neural

349 response: linear summation *vs.* simultaneous stimulation, 6.39±1.36 μV *vs.* 8.76±1.23 μV,

350 *P<0.05, n=8; behavioral response: linear summation *vs.* simultaneous stimulation,

351 0.39±0.33 licks/s *vs.* 1.02±0.32 licks/s, *P<0.05, n=8; Mean±SEM; two-tailed paired *t*-

352 test); the simultaneous stimulation induced nonlinear facilitation of responses. AEPs and

353 mean PSTHs of licking behavior elicited by auditory and laser stimulation with supra-

354 threshold intensity were described in Fig. S7.

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Materials and methods

Animals and their maintenance

In total, fifty-seven experimentally naive adult Mongolian gerbils (twenty-six females, thirty-one males; *Meriones unguiculatus*) aged 2–9 months were used. Each gerbil was bred with two to four other gerbils in a cage measuring 20 (W) × 40 (L) × 17 cm (H), with free access to food and water. The temperature in the animal room was maintained between 22 and 23ºC, with approximately 50% relative humidity and a 13-hr light: 11-hr dark cycle. All experimental procedures were performed according to the guidelines established by the Ethics Review Committee of Doshisha University. The Animal Experimental Committee of Doshisha University approved the experimental protocols.

Animal surgery

All surgical procedure was performed under anesthesia using an intramuscular combined injection of ketamine (47 mg/kg) and xylazine (9.3 mg/kg). Maintenance doses of ketamine (17.5 mg/kg) and xylazine (7.0 mg/kg) were injected every 30–50 min or if the animals showed signs of increasing arousal (e.g., voluntary whisker movement). The body temperature of gerbils was maintained using heating pads placed beneath the animals.

The parietal and temporal sides of the skin of the subject's head were retracted after

shaving the fur over the skin. Both sides of the temporal muscle were eliminated after applying a local anesthetic (xylocainegel; Aspen Japan, Tokyo, Japan). The skull was carefully cleaned with 0.3% sodium hypochlorite solution and saline. The temporal skull 3.0 mm posterior from the bregma and 4 mm ventral to the lamda-bregma plane was drilled to make a small hole (diameter: 1mm) for a recording electrode. A small hole was also made in the dorsal skull 2 mm anterior to the bregma and 1 mm lateral to the midline for reference. A reference and recording electrodes (Nilaco, Tokyo, Japan; diameter: 0.13 mm; impedance < 20 kΩ) were inserted into these holes and temporally fixed approximately 1 mm depth from the surface of the cortex with acrylic glue. Electrode insertion sites were determined based on the brain atlas in Mongolian gerbils (*1*) and our previous study (*2*). After the temporal fixation of the electrodes with acrylic glue, the whole exposed skull was covered with dental resin (Super bond C&B; Sun Medical, Shiga, Japan). A V-shaped metal plate (F-911; Hilogik, Osaka, Japan) was attached to the dental resin over the skull using dental cement (Provinice; Shofu, Kyoto, Japan), allowing the subjects to be head-fixed during the experiment.

The left side of the pinna was detached to provide a clear view of the tympanic membrane and outer canal. An incision of the muscle and skin from the shoulder to the jaw exposed the left side of the bulla, and a hole with 1 mm diameter was made on the

bulla. A silver electrode to record cochlear responses (Nilaco, Tokyo, Japan; diameter: 0.13 mm; impedance < 20 kΩ) was inserted into the hole and hooked onto the bony rim of the round window. The electrode was stabilized with a bulla and skull using acrylic glue and dental cement (Provinice; Shofu, Kyoto, Japan).

All subjects were singly housed after the surgery and provided a recovery period for at least two days before training began. Animal surgery was performed following the same procedure as our previous studies (*3, 4*).

Auditory stimuli

Click train with a repetition rate of 4 kHz were used as auditory stimuli. The click train consisted of 100 μs rectangular pulses, and the duration of the auditory stimulation was 500 ms. The auditory stimulus was presented via the speaker (AS01008MR-2-R, Pui audio, Ohio, America), which was placed at 4.5 cm from the gerbil's left ear with a vertically 45° angle. The auditory stimulus was generated using a digital-to-analog converter (Octa-Capture, Roland, Shizuoka, Japan) with a 192 kHz sampling rate. The signal was intensified with an amplifier (A-10, Pioneer, Tokyo, Japan). The sound pressure level was adjusted from 20 to 95 dB SPL using a microphone (Type 7016, Aco, Tokyo, Japan)

Laser stimuli

A repetitive pulsed infrared laser was used to apply the infrared laser stimuli. The individual laser pulse had 100 μs rectangular shape in intensity, and the inter-pulse interval was 0.25 ms. The pulsed infrared laser was created with a diode laser stimulation system (BWF-OEM, B&W TEK, Delaware, USA) with a wavelength (λ=1871) in line with previous studies (*3-8*). The duration of the repetitive pulsed infrared laser was 500 ms. The voltage commands for laser stimuli were generated with a digital-to-analog converter (Octa-Capture, Roland, Shizuoka, Japan). The laser was presented via an optical fiber (diameter: 100 μm; NA, 0.22) inserted mediolaterally into the left outer ear canal with the tip angled rostrally by 10° and dorsally by 5° using a micromanipulator (MM-3; Narishige, Tokyo, Japan) in awake head-fixated subjects. The optic fiber tip was fixated at approximately 0.7 mm (the distance between the cochlea and fiber tip was about 2.2 mm) in front of the tympanic membrane to ensure that the cochlea was irradiated transtympanically from the outer ear as described in our previous reports (*3, 4, 7, 8*). The laser-beam profile in each pulsed laser at the cochlea (i.e., 2.2 mm from the fiber tip) was measured as in our previous studies (*3, 4*). The radiant energy was measured using a digital power meter (PM100D; Thorlabs, Tokyo, Japan) with a thermal power sensor (S302C; Thorlabs, Tokyo, Japan) and calibrated between 0.1 and 13.2 mJ/cm^2. The beam width (full width at half the maximum diameter) at the cochlea was calculated with an

InGaAs-biased detector (DET10D/M; Thorlabs, Tokyo, Japan) and was measured as 2.2 mm.

Visual stimuli

A white light-emitting diode (LED) (diameter: 5 mm) was used for the visual stimulation. The LED was placed 1 cm from the gerbil's left eye in the horizontal plane. The voltage command for the visual stimulus was controlled by a digital-to-analog converter (Octa-Capture, Roland, Shizuoka, Japan) with a 192 kHz sampling rate. The light stimulation was applied for 500 ms, and light intensity was calibrated at 96 lux using a digital lux meter (GL-03; be-s, Osaka, Japan).

Synchronous presentation of auditory and laser stimuli

Simultaneous presentation of click train and pulsed laser train was performed to assess the feasibility of simultaneous presentation of auditory and laser stimulation. The stimulus feature of the simultaneous stimulation was the same as each auditory and laser stimulation except for simultaneously presenting both stimuli.

Apparatus in the behavioral experiment

Behavioral experiments and electrophysiological recording were performed in the same soundproof box 60 (W) × 60 (L) × 60 cm (H), following our previous study (*4*). Each subject was positioned on a custom-designed covered platform in which a copper

sheet was set. The subject's heads were stabilized by clamping the V-shaped metal plate attached to the heads with the clamper installed on the sidebars near the platform. A metal drinking spout was fixed 1 mm in front of the mouth of the subject, and the position of the drinking spout was adjustable to detect licking behavior easily. A touch sensor (DCTS-10; Sankei Kizai, Tokyo, Japan), connected between the drinking spout and the copper sheet of the stage, recorded the timing of licks at 2000 samples/s. Voltage change from the touch sensor (i.e., licking timing) was captured by Arduino Uno (Arduino, Ivrea, Italy), controlled by Python 3.7 on Spyder 501 and stored on a computer. All behaviors were recorded using a video camera (HD-5000; Microsoft, Washington, USA).

Training session

After at least two days of recovery from surgery, the gerbils were water-restricted in their home cage. The training session typically comprised 250–400 trials daily. Head-fixed animals could voluntarily lick the spout throughout the behavioral experiment. A 3.2 μl drop of water (unconditioned stimulus: US) was delivered through the tube 678 ± 32 ms (Mean±SD, n=120) after the onset of auditory, laser, or visual stimulation (i.e., conditioned stimulus: CS). Inter trial interval was randomly varied between 7 and 13 s. The timing of US and CS was controlled by MATLAB (MathWorks, Massachusetts, USA) and a microcontroller (Arduino Uno; Arduino, Ivrea, Italy) with a custom-made

relay circuit. The change in licking behavior with the head-fixed Pavlovian conditioning is described in Fig S1 and S2.

The procedure of the test session

After animals showed high licking rates following the CS without delivering the US, a test session was performed. The test session comprised 280 rewarded and 120 non-rewarded trials (i.e., catch trials). In the rewarded trial, CS and US were delivered in the same manner as the training session. Rewarded trials functioned as maintenance of a high motivational state in subjects. The catch trials assessed the subjects' perceptions without reward feedback. Six kinds of stimuli were presented in the catch trial; the behavioral response to each stimulus was tested 20 times. Further details in each stimulus condition are provided in Fig S3 and S4.

Recording and analysis of the behavioral data

An averaged peri-stimulus time histogram (PSTH) of licking behavior was used to evaluate the time course of behavioral responses. The PSTHs were calculated in a 100 ms bin size. To smooth the PSTHs, three points moving average (i.e., 300 ms window) was performed. For quantifying behavioral response, peak licking rates, durations, peak periods, latencies, and delta licking rate (DLR) were extracted from the PSTHs as in our previous study (*4*). Peak licking rates and periods were defined as the licking rate and

time when the licking rate reached the maximum value within 4 s after stimulus onset, respectively. The baseline licking rate was defined as the pre-stimulus licking rate obtained for 1 s, and duration was quantified as the temporal length of the PSTH >50% of the licking rate from the mean baseline licking rate to the peak licking rate. Latency was calculated by measuring the time between stimulus onset and the behavioral response reaching the mean plus three standard deviations of the baseline licking rate. The DLR was defined as the difference between the mean baseline licking rate and the post-stimulus licking rate (analysis range: 0.2–1.2 s) to evaluate the response amplitude. The analysis range of post-stimulus licking behavior was determined by latency data of the behavioral response (Fig. S5) and our previous study (*4*).

Electrophysiological recording in the behavioral experiment

The cochlear response was recorded in awake subjects during the behavioral experiment. The cochlear response was amplified 1000 times using a bio-amplifier (MEG-1200; Nihon Kohden, Tokyo, Japan). An analog-to-digital converter (Octa-capture; Roland, Shizuoka, Japan) at a sampling rate of 96,000 Hz was used to store the electrophysiological data on a computer.

The recorded signal was processed utilizing a band-pass filter (500–3500 Hz) to minimize the summating potentials and cochlear microphonics (CMs) and to extract the

compound action potential (CAP) of the cochlear nerves. The amplitude of CAP signals was defined as the peak-to-peak voltage between the first minimum (N1) and maximum (P1) of each CAP response.

In the auditory masking experiment, the peak of CAP signals was hard to be defined because CM elicited by white noise (i.e., masker) was extremely massive compared to CAP; the band pass filter could not completely remove CM. Therefore, the change in root means square (RMS) amplitude between pre- and post-signal onset was measured (i.e., diff RMS amplitude). The signal onset was determined by visual inspection using MATLAB (MathWorks, Massachusetts, USA). Pre- and post-CAP signals for 2 ms from the signal onset were obtained to calculate the relative voltage change in CAP triggered by auditory and laser stimulation.

Auditory evoked potential (AEP) recorded from the anesthetized subjects

For investigating the effect of the synchronous presentation of auditory and laser stimulation on central nervous response, an AEP was recorded from the right auditory cortex using inserted silver electrode (Nilaco, Tokyo, Japan; diameter: 0.13 mm; impedance < 20 kΩ) in anesthetized animals with ketamine (47.0 mg/kg; i.m.) and xylazine (9.3 mg/kg; i.m.). The AEP signal was amplified 1000 times using a bio-amplifier (MEG-1200; Nihon Kohden, Tokyo, Japan) and stored in a computer through

an analog-to-digital converter (Octa-capture; Roland, Shizuoka, Japan) at a sampling rate of 192,000 Hz. Maintenance doses of ketamine (17.5 mg/kg; i.m.) and xylazine (7.0 mg/kg; i.m.) were injected every 30 min during the recording. Each subject was placed on the same covered elevated platform in the soundproof box as in the behavioral experiment. Portable warm packs were attached to the cover of the platform, and a small pee pad was placed beneath the subject to maintain the subject's temperature.

To examine the feature of the laser interactions with the auditory-evoked response, both clicking sound (35 dB pe. SPL) and pulsed laser (1.1 mJ/cm^2) were presented, and the relative timing between them was changed; the auditory stimulus onset varied 0, 5, 10, 20 ms either before or after the laser stimulation. Clicking sound and pulsed laser were generated from the digital-to-analog converter (Octa-Capture, Roland, Shizuoka, Japan) with a 192 kHz sampling rate. The voltage command of auditory and laser stimulation was controlled by stereo channels of the digital-to-analog converter to manipulate their relative timing precisely. The AEP signals between 50 and 300 ms from laser stimulus onset were captured, and the root means square (RMS) of the extracted signal was calculated as the response amplitude of the AEP signals. The analysis range was defined based on previous studies (*9-13*) to evaluate the auditory cortical activity, which generally plays an important role in perception and to prevent analyzing auditory

stimulus artifacts. Auditory evoked potential (AEP) depending on the difference in the relative timing of stimulus onset was described in Fig S9.

In addition, simultaneous presentation of click train (20, 30, 40, 50 dB SPL) and pulsed laser train (0.1, 0.2, 1.1, 6.9 mJ/cm^2) was performed to assess the feasibility of simultaneous presentation of auditory and laser stimulation. These sound pressure levels and radiant energy were selected as sub-, near-, or supra-threshold intensity based on previous studies (*2, 3, 7, 8, 14*). The AEP responses elicited by simultaneous presentation of auditory and laser stimulation, which induced almost the same AEP amplitude (i.e., higher AEP amplitude induced by auditory or laser stimulation was not more than twice the lower response amplitude), were recorded. The degree of nonlinear amplification depending on response amplitude elicited by mono stimulus was investigated. The response amplitude induced by mono-stimulation was defined as the median of response amplitude induced by auditory and laser stimulation alone. The obtained data were separated based on the response amplitude induced by mono-stimulation because the tendency of nonlinear amplification was obviously different between the group in which no observable response (amplitude: 3.5 μV or lower) and conspicuous response (amplitude: 20 μV or higher) were found by the presentation of mono-stimulus. The mono-stimulus intensities that elicit no observable response and conspicuous response

were defined as sub- or near-threshold and supra-threshold intensity, respectively.

Supplemental Figure

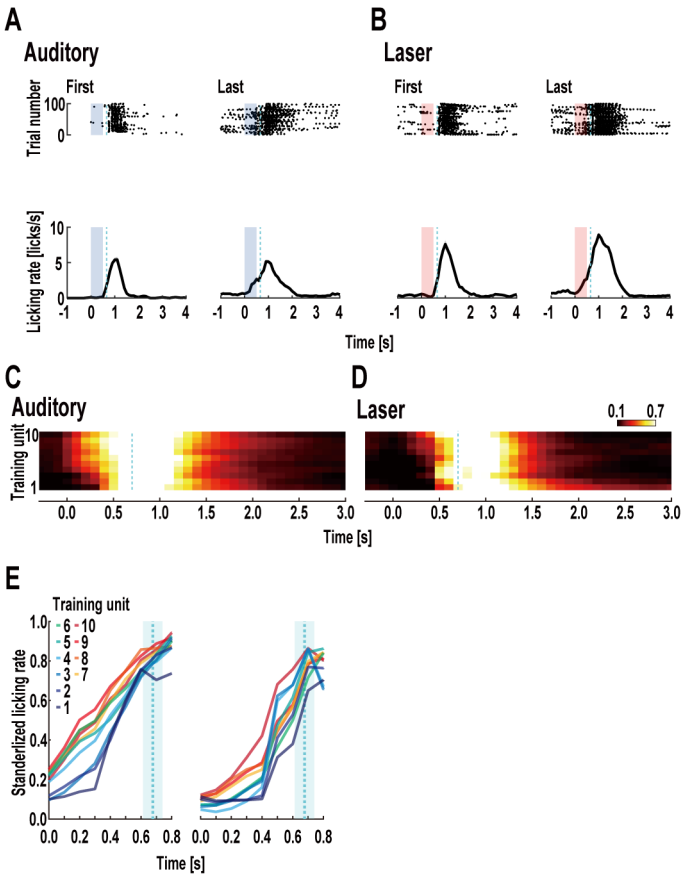

Fig. S1: Learning curves for licking behavior elicited by auditory and laser stimulation. Auditory (A)- and laser (B)-induced licking behavior in the first (1–100 trials) and last (901–1000 trials) training units. One training unit was composed of 100 trials. Individual trials (top) and mean peri-stimulus time histogram (PSTH) of the licking behavior (bottom) elicited by auditory and laser stimulation are described. The blue and red zones indicate auditory and laser stimulation periods. The blue dotted lines show the reward timing. Black plots in the top figure show licking timing. In the first training unit, auditory- and laser-evoked licking responses were observed after presenting the US,

while these licking behaviors were also recorded prior to the presentation of the US in the last training unit. (C) Heat map of the average PSTH of the licking behavior in the rewarded trial of each training unit (auditory: n=13; laser: n=12). Each row is a different training unit. The blue dotted lines show the reward timing. All PSTH was standardized by the peak licking rate in each training unit. As the training progressed, the standardized auditory- and laser-induced licking rates were observed earlier by degree after the CS onset. (D) Change in the average auditory (n=13)- and laser (n=12)-CR in each training unit. The blue dotted lines and areas show the mean reward timing and its 2SD. The standardized licking rates until 0.6 s after the stimulus onset were measured as CR. The gradual increases in CR amplitude were observed session after session. (E) The average amplitude of auditory (n=13)- and laser (n=12)-conditioned response. Gray areas show the standard error of the mean.

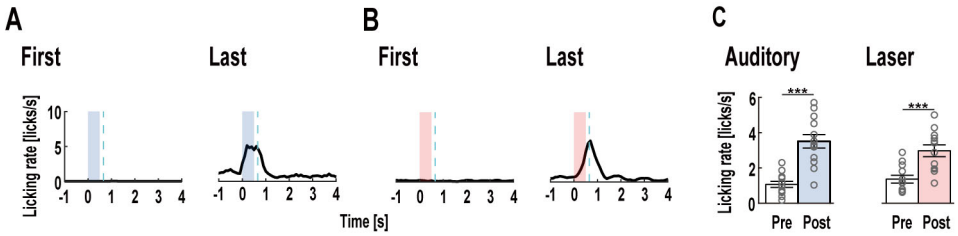

Fig. S2: Licking behavior transition in catch trial by Pavlovian conditioning with auditory and laser stimulation. Auditory (A)- and laser (B)-induced raster plots (upper) and an average PSTH of the licking rate (bottom) on the first and last day of conducting catch trials (1-120 trials). The blue and red zones indicate auditory and laser stimulation periods. The blue dotted lines show the reward timing in the rewarded trial. Black plots show licking timing. Catch trials were performed to monitor the association between the CS and US. Synchronous increments of licking rate with the auditory and laser stimulation were not obtained on the first day of the catch trial. After the training was completed, the synchronous licking behaviors with CS were observed. (C) Mean licking rate with auditory (n=13) and laser (n=12) stimulation on the last training day. White bars show the mean licking rate for 1 s before the stimulus onset. Blue and pink bars indicate the mean licking rate between 0.2 s and 1.2 s. Gray open circles show individual data. The mean licking rates after stimulus onset were significantly higher than that before stimulus onset in both auditory and laser stimulation.

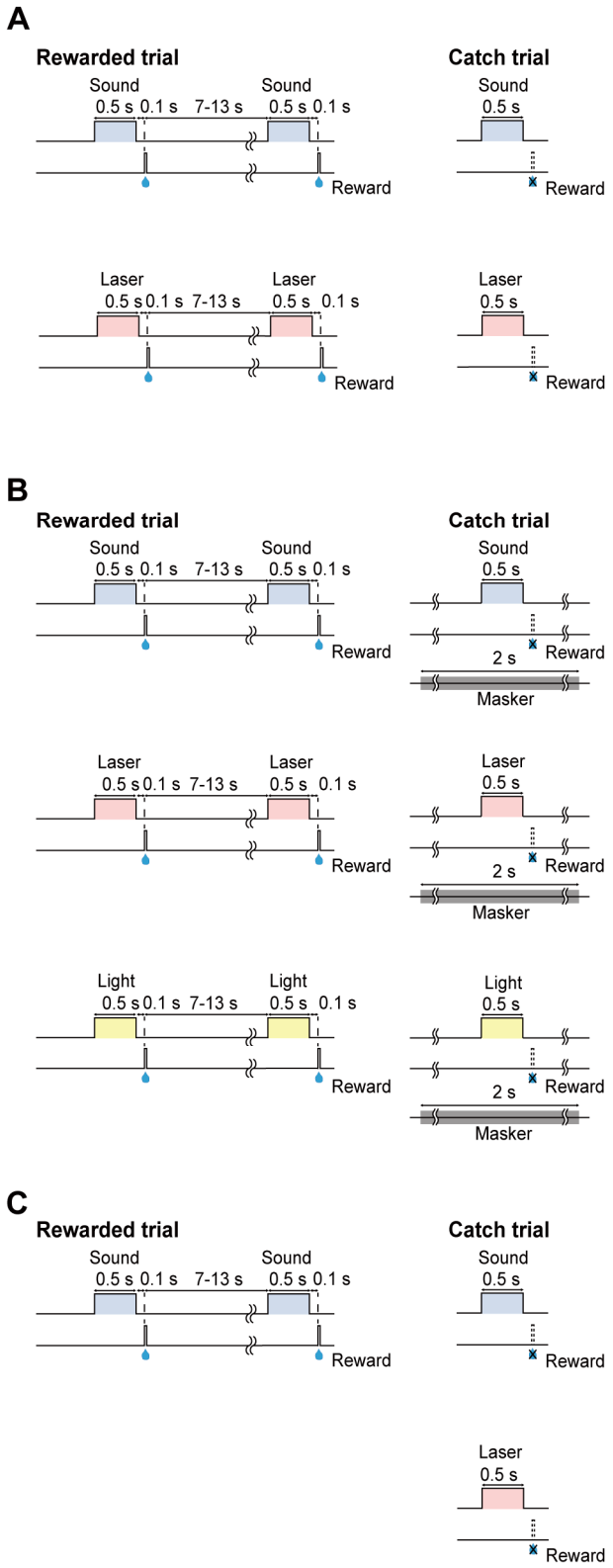

Fig. S3: Schematic diagram of conditioned (CS) and unconditioned (US) stimulus

presentation in the mono-stimulus experiment. The behavioral task of intensity dependence of auditory- and laser-evoked response (A), the effect of auditory masking on auditory- and laser-evoked response (B), and the assessment of stimulus generalization (C) were shown. The timing of conditioned (CS) and unconditioned (US) stimuli in the rewarded trial (left) and catch trial (right) are described. In (A), fifteen auditory (70 dB SPL)- and six laser (11.7 mJ/cm^2)-trained animals were used in the experiment. In the rewarded trial (left), click train (70 dB SPL) or pulsed laser train (11.7 mJ/cm^2) was presented to the auditory- and laser-trained animals, respectively. In the catch trials (right), sound pressure level and radiant energy of CS were varied (auditory: 20, 35, 50, 65, 80, or 95 dB SPL; laser: 0.1, 1.1, 3.1, 6.9, 10.2, or 13.2 mJ/cm^2). In (B), the experiment used six auditory- (80 dB SPL), six laser- (13.2 mJ/cm^2), and four visual- (96 lux) trained animals. In the rewarded trial, click train (80 dB SPL), pulsed laser train (13.2 mJ/cm^2), or LED light (96 lux) was presented to the auditory-, laser-, or visual-trained animals, respectively. For investigating the effect of auditory masking on the physiological and behavioral response induced by auditory, laser, and visual stimulation, the white noise (700–44,800 Hz) of 0, 35, 50, 65, 80, or 95 dB SPL for 2 s was presented during auditory, laser, or visual stimulus period of catch trials. In (C), all animals (n=11) were trained by auditory (70 dB SPL) stimulation. In the rewarded trials, a click train (70 dB SPL) was

presented. Auditory (20, 50, 80 dB SPL) and laser (0.1, 6.6, 13.2 mJ/cm^2) stimuli were introduced in the catch trials.

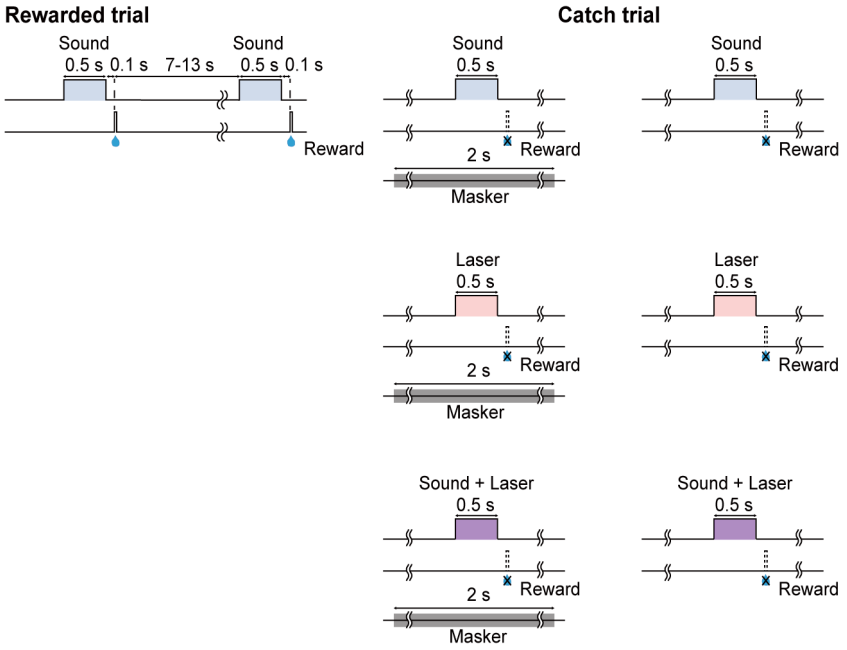

Fig. S4: Illustration of the behavioral task in evaluating the feasibility of simultaneous presentation of auditory and laser stimulation. The timing of conditioned (CS) and unconditioned (US) stimuli in the rewarded trial (left) and catch trial (right) are described. Eight subjects were trained with auditory (80 dB SPL) and laser (13.2 mJ/cm^2) stimulation. White noise (70 dB SPL) was presented during the stimulus period in the same way as the auditory masking experiment to assess the efficiency of simultaneous stimulation in a quiet and noisy environment. An auditory (80 dB SPL) stimulus was introduced in the rewarded trials. In the catch trials, auditory (80 dB SPL), laser (13.2 mJ/cm^2), or simultaneous stimulation was presented with or without background white noise.

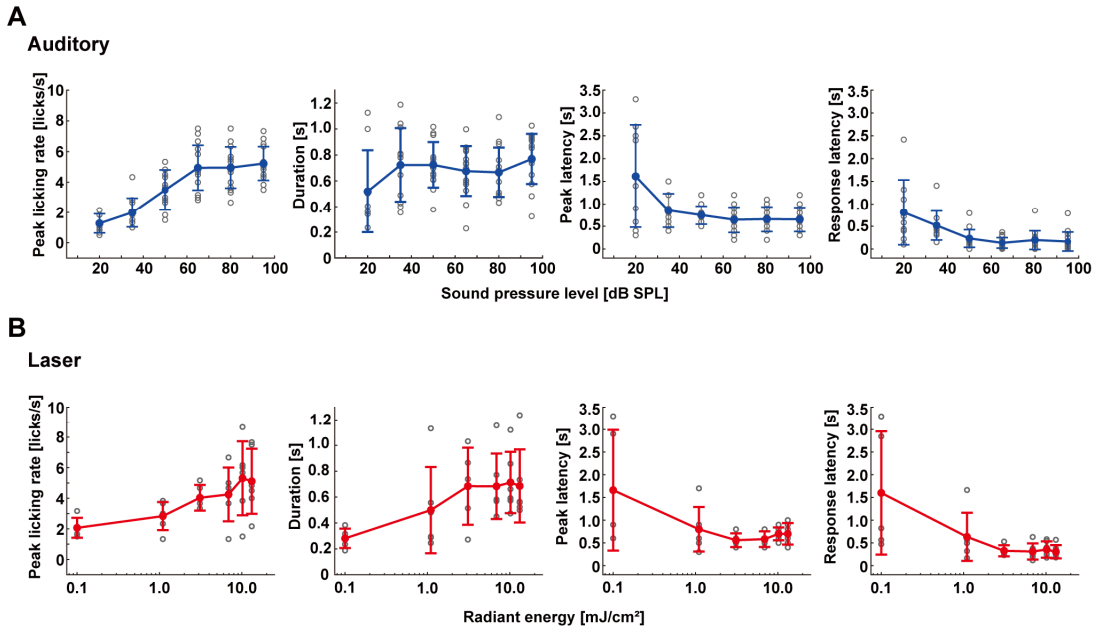

Fig. S5: Change in the feature of behavioral response by modulating sound pressure level and radiant energy. Intensity dependence of peak licking rate (leftmost), duration (left), peak latency (right), and response latency (rightmost) with auditory (A) and laser (B) stimulation. As the sound pressure level increased from 20 to 95 dB SPL, the peak licking rate rose from 1.28±0.63 to 5.22±1.10 licks/s; duration was increased from 0.52±0.32 to 0.77±0.19 s; the peak latency was decreased from 1.61±1.13 to 0.65±0.27 s; response latency was reduced from 0.81±0.72 to 0.16±0.21 s. As radiant energy increased from 0.1 to 13.2 mJ/cm^2, the peak licking rate rose from 2.07±0.65 to 5.11±2.13 licks/s; duration was increased from 0.28±0.07 to 0.69±0.28 s; the peak latency was decreased from 1.66±1.33 to 0.70±0.24 s; response latency was reduced from 1.60±1.36 to 0.31±0.15 s. The properties of auditory-evoked behavioral response were in line with

our previous studies (*4*) and were similar to that of laser-evoked behavioral response.

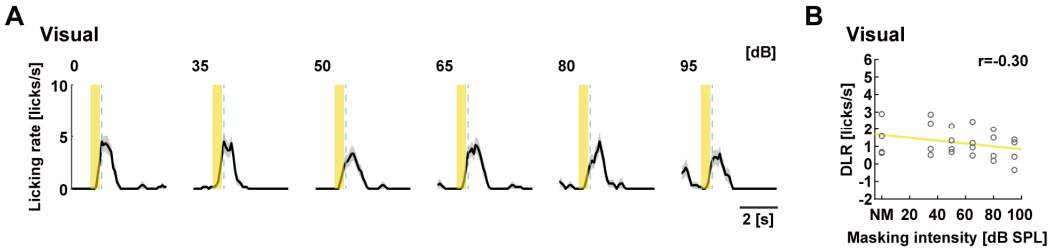

Fig. S6: The effect of the auditory masking on BR evoked by visual stimulation. This investigation was performed as a control condition to ensure that the presence of intensive white noise did not cause behavioral disturbance as well as interference with auditory input. (A) The mean PSTH of licking behavior elicited by visual stimulation in masking experiment. Yellow areas show stimulus duration. Blue dotted lines show reward timing in a training session. (B) Correlation between masking intensity and visually evoked DLR. The result of the control experiment showed that a significant decrement in visual-evoked DLR was not observed, as the sound pressure level of white noise increased (r=0.30, p=; Pearson's correlation analysis); therefore, auditory masking in this experiment mainly interrupted the auditory-related perception.

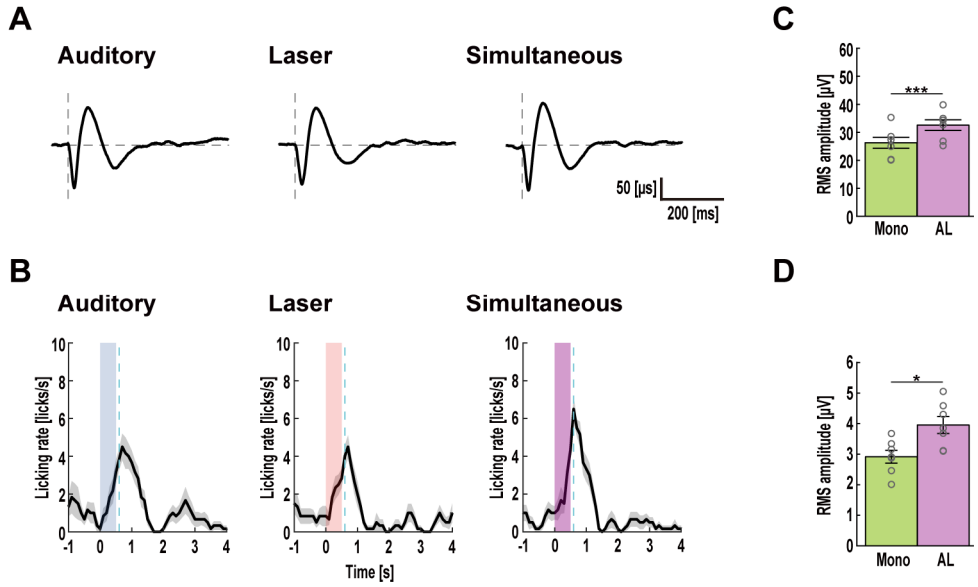

Fig. S7: The interaction between auditory and laser stimulation with the supra-threshold intensity. Comparing AEP (A) and PSTH of licking behavior (B) induced by suprathreshold auditory, laser, and simultaneous stimulation. In (A), vertical and horizontal dotted gray line show stimulus onset and 0 μV, respectively. Blue dotted lines in (B) show the expected reward timing. Gray areas show SEMs of each trial. The blue, red, and purple zones indicate the auditory, laser, and simultaneous stimulating periods. Response amplitude by mono-stimulus and simultaneous stimulation in neural (C) and behavioral (D) responses were described. When we simultaneously presented supra-threshold auditory and laser stimulation, nonlinear facilitation was not observed in neural and behavioral responses, unlike simultaneous stimulation with sub- or near-threshold intensity in Fig.4. However, simultaneous presentation of auditory and laser stimulation with supra-intensity induced significantly higher response amplitude than mono

stimulation; simultaneous presentation of supra-threshold auditory and laser stimulation induced significantly greater neural and behavioral response than mono stimulation although the degree of amplification caused by supra-threshold simultaneous stimulation is less than a sub- or near-threshold simultaneous stimulation.

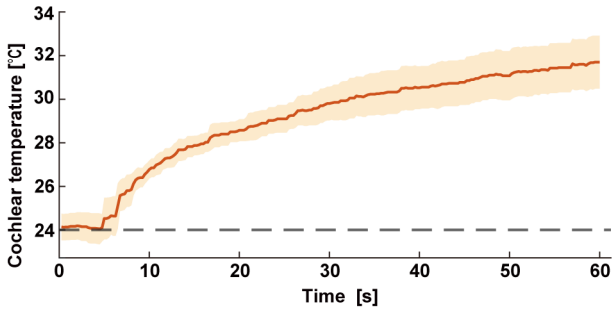

Fig. S8: Time course of the thermal build-up of the cochlear surface induced by continuous 13.2 mJ/cm^2 laser irradiation. A bold orange line and area show the average temperature in each cochlea and its standard deviation, respectively (n=4). A gray dotted line indicates room temperature (i.e., 24°C). The temperature of the cochlear surface started increasing approximately at 5 s after the laser onset. Therefore, 500 ms laser irradiation with 13.2 mJ/cm^2 (i.e., maximum intensity in this experiment) might not cause thermal elevation of the cochlear surface.

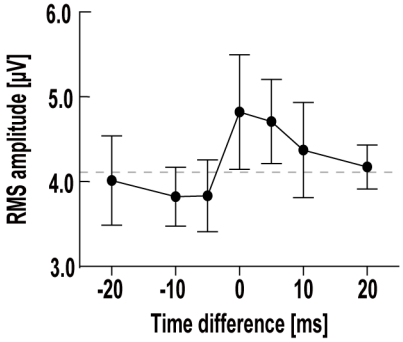

Fig. S9: Auditory evoked potential (AEP) depending on the difference in the relative timing of stimulus onset. The effect of the relative timing of the auditory and laser stimulation on AEP and their response amplitudes is described. Gray dotted lines are baseline amplitude (horizontal line), calculated by averaging the root mean square (RMS) amplitude of the pre-stimulus electrophysiological signal obtained for 50 ms. Blue, red purple lines show stimulus onset of auditory, laser, and simultaneous stimulus. When the auditory stimulus was presented before the laser stimulus, the RMS amplitude decreased as the time difference of the stimulus onset was diminished (from -20 to -5 ms). The peak of RMS amplitude was observed at a simultaneous stimulation without delay. Then the amplitude decreased as the auditory stimulation lag increased (5 to 20 ms).

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