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Deletion of N-type Ca^{2+} Channel $Ca_v2.2$ Results in Hyperaggressive Behaviors in Mice*[S]

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Voltage-dependent N-type Ca^{2+} channels play important roles in the regulation of diverse neuronal functions in the brain, but little is known about its role in social aggressive behaviors. Mice lacking the α1B subunit ($Ca_v2.2$) of N-type Ca^{2+} channels showed markedly enhanced aggressive behaviors to an intruder mouse in the resident-intruder test. The dorsal raphe nucleus (DRN), which contains serotonin neurons, is known to be involved in aggression in animals. We thus examined the DRN neurons in the $Ca_v2.2$-deficient ($Ca_v2.2^{-/-}$) mice. Microinjection of ω-conotoxin GVIA, an N-type Ca^{2+} channel-specific blocker, into the DRN of wild type mice resulted in escalated aggression, mimicking the phenotypes of $Ca_v2.2^{-/-}$. Electrophysiological analysis showed increased firing activity of serotonin neurons with a reduced inhibitory neurotransmission in the $Ca_v2.2^{-/-}$ DRN. $Ca_v2.2^{-/-}$ mice showed an elevated level of arginine vasopressin, an aggression-related hormone, in the cerebrospinal fluid. In addition, $Ca_v2.2^{-/-}$ mice showed an increase of serotonin in the hypothalamus. These results suggest that N-type Ca^{2+} channels at the DRN have a key role in the control of aggression.

Voltage-dependent Ca^{2+} channels play important roles in the regulation of diverse neuronal functions, including neurotransmitter release, regulation of cell membrane excitability, and control of gene expression. Ca^{2+} influx via N-type Ca^{2+} channels has a crucial role in controlling the release of excitatory and inhibitory neurotransmitters at presynaptic terminals in central synapses (1). Recently, genetically engineered mice lacking N-type Ca^{2+} channel α1B subunit have been developed and used in experiments to clarify *in vivo* functions of N-type Ca^{2+} channels. Although previous analyses of $Ca_v2.2$-deficient ($Ca_v2.2^{-/-}$)[4] mice have revealed physiological roles of N-type Ca^{2+} channels in various behaviors (2–6), there was no study of the role of N-type Ca^{2+} channels in aggression.

It is believed that most social animals possess neural mechanisms for the control of aggression, which is essential for maintenance of beneficial relationships among members in a community. Aggression is considered a complex social behavior influenced by both internal (*e.g.* hormones or genes) and external stimuli (*e.g.* drive, TV, and frustration etc.) (7). Deficit of the control mechanisms for suppression of aggression might be implicated in the development of violence in various psychiatric disorders, such as attention deficit/hyperactivity disorder (ADHD) and personality disorders (8, 9).

The modulation of aggression has been associated with a change in the central serotonin (5-HT) system at the dorsal raphe nucleus (DRN) (10–14). In the DRN, the 5-HT neuronal activity showing a slow and regular firing pattern (15–17) is known to be influenced by γ-aminobutyric acid (GABA), 5-HT_{1A} autoreceptor, and noradrenergic input (17). Furthermore, the modulation of these signals in the DRN affected aggression behaviors in animals (10–13, 18, 19).

It has been shown that the $Ca_v2.2$ is highly expressed in the DRN of adult rats (20). However, the role of $Ca_v2.2$ in the DRN has not been examined, nor has the involvement of $Ca_v2.2$ in the regulation of aggression. Therefore, we have characterized aggression behaviors of $Ca_v2.2^{-/-}$ mice and investigated the properties of the $Ca_v2.2^{-/-}$ DRN neurons. Interestingly, the $Ca_v2.2^{-/-}$ mice exhibited an enhanced aggressive behavior and a reduced inhibitory transmission in the DRN. In addition, $Ca_v2.2^{-/-}$ mice showed an increased level of arginine vasopressin (AVP) in the cerebrospinal fluid (CSF), together with an increase of 5-HT in the hypothalamus. Our results suggest a possibility that the N-type Ca^{2+} channel plays a critical role in the suppression of aggressive behaviors.

EXPERIMENTAL PROCEDURES

Animals—All of the animals were handled in accordance with the animal care and use guidelines of the Korea Institute of

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental data, Figs. S1 and S2, and Table S1.

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[4] The abbreviations used are: $Ca_v2.2$, N-type Ca^{2+} channels α1B subunit; DRN, dorsal raphe nucleus; 5-HT, serotonin; AVP, arginine vasopressin; GABA, γ-aminobutyric acid; CSF, cerebrospinal fluid; IPSC, inhibitory postsynaptic current; EPSC, evoked postsynaptic current; HPLC, high pressure liquid chromatography; ADHD, attention deficit/hyperactivity disorder; 5-HIAA, 5-hydroxyindolacetic acid.

Science and Technology. The animals were housed in a temperature- and humidity-controlled environment with free access to food and water under a 12-h light/12-h dark cycle. Mice lacking the α1B subunit ($Ca_v2.2$) were established previously (2). For this study, homozygotes ($Ca_v2.2^{-/-}$) in the F1 (C57BL/6J × 129S4/SvJae) background were produced by crossing heterozygous 129 co-isogenic mice ($Ca_v2.2^{+/-}$) with heterozygous N12 C57BL/6J mice ($Ca_v2.2^{+/-}$). The $Ca_v2.2^{-/-}$ male mice (10–15 weeks old) in the F1 background were used for behavioral tests, and wild type littermates served as control. All of the behavioral experiments were performed and videotaped by one investigator, and the tape was interpreted by another investigator blinded to the genotypes of the animals used in the experiments.

Resident-Intruder Test—The test was based on a previously described protocol (21). The isolation-induced resident-intruder aggression test was conducted by introducing a small, experimentally naïve male C57BL/6J mouse (5–6 weeks old) that had been housed in groups of five into a clear polycarbonate cage (27 × 21 × 17 cm) of a resident male mouse (10–15 weeks old) that had been housed in isolation for at least 4 weeks without a change of bedding for 7 days before testing. The behavior of the resident mouse was videotaped, and offensive aggression was measured by determining the latency to the first attack and total number of bite attacks by the isolated resident mouse during 15 min of exposure to the experimental naive male C57BL/6J intruder mouse. If an animal did not make a bite attack, the latency to the first attack was recorded as 900 s (test duration), and all the other attack scores were recorded as zero.

Water Competition Test—The water competition test used was based on a previously described method (22). Male mice of both genotypes of equal weight were paired and housed together in a same cage. After 6 days, the animals were deprived of water for 23 h. One water bottle was introduced with a shielded spout so that only one animal could drink at a time. The time (in seconds) of spout possession and water consumption were recorded for 2 min. The animal with the longest duration of water consumption and spout possession was considered to be the dominant animal.

Intra-raphe Injection and Histology—Wild type male mice were anesthetized with Avertin® (2,2,2 tribromoethanol; 0.2 ml/10 g of 20 mg/ml solution), and a 26-gauge guide cannular (Plastics One) for microinjection of the N-type Ca^{2+} channel blocker, ω-conotoxin GVIA (ω-GVIA), was stereotaxically placed into the DRN. The cannular was implanted at anterior-posterior = −4.5 mm from the bregma; lateral = ±1.2 mm; ventral = −4.0 mm; tilted 22.5 degree, as calculated from the mouse brain atlas (23). After 10 days, the animals were injected either with vehicle (0.9% NaCl containing 1 mg/ml cytochrome C) alone or with ω-GVIA (9 ng/0.2 μl/5 min, Alomone) into the DRN 1 h before the resident-intruder test. After the experiments, the mice were deeply anesthetized by injection of Avertin®, and the brains were removed, fixed in 4% paraformaldehyde at 4 °C for at least 48 h, and stored in a 30% sucrose solution until being sliced on a freezing microtome (30-μm-thick sections) (23). The brains were lightly stained with cresyl violet for histological verification of needle placement. All of the injection sites included in the statistical analyses were referenced to the mouse brain atlas (24) and illustrated in Fig. 2.

Extracellular Recording of 5-HT Neurons—Mice (8–12 weeks old) were decapitated, and coronal 400-μm brain stem slices containing the DRN were prepared in oxygenated cold artificial CSF (124 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, and 10 mM glucose, pH 7.4). After incubation for 1 h at room temperature, a single slice was submerged in a recording chamber and continuously superfused with oxygenated artificial CSF (34 °C; 95% O_2/5% CO_2) (25). Firing was recorded in the DRN using a borosilicate glass microelectrode (8–10 MΩ) filled with 2 M NaCl and evoked by adding the α_1-adrenoreceptor agonist phenylephrine (3 μM) (17). The cells were identified as putative 5-HT neurons according to the following criteria: biphasic action potentials, long spike width (>2 ms), slow and regular pattern of discharges, and reversible inhibition by 5-HT (100 μM) (15, 17). Signals were amplified 10,000-fold and filtered at 300~3000 Hz by a Cyberamp-402 amplifier (Axon Instruments), digitized at a 10-kHz sampling rate, and stored in a computer (Digidata; Axon Instruments). The number of spikes was quantified using Mini-Analysis software (Synaptosoft).

Whole Cell Patch Clamp Recording of 5-HT Neurons—Mice (2.5–3.5 weeks old) were decapitated, and brain stem slices (250~300 μm) containing the DRN were prepared as described above for extracellular recordings, and the methods of patch clamp recording were described in our previous report (25). Pipettes (3–4 MΩ) were filled with the internal solution for patch clamp recording (140 mM KCl, 0.1 mM $CaCl_2$, 4.6 mM $MgCl_2$, 0.1 mM EGTA, 10 mM HEPES, 4 mM Na-ATP, and 0.4 mM Na-GTP, adjusted to pH 7.3 with KOH). The DRNs were visually identified by their position along the midline in the slice and by their morphology. Although the staining of 5-HT or tryptophan hydroxylase is needed exactly to identify a 5-HT neuron, cells, at least, that meet the electrophysiological criteria of a 5-HT neuron were recorded (supplemental data). For IPSC and EPSC measurement, the cells were voltage-clamped at −60 mV. GABA receptor-mediated sIPSC and eIPSC was isolated and recorded in the presence of 6-cyano-7-nitroquinoxaline-2.3-dione (10 μM, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist), DL-2-amino-5-phosphonovaleric acid (50 μM, *N*-methyl-D-aspartate receptor antagonist), and CGP 55845 (5 μM, a selective $GABA_B$ receptor antagonist). sEPSC and eEPSC were measured in the presence of bicuculline (10 μM, $GABA_A$ receptor antagonist) and CGP 55845. For the eIPSC and eEPSC experiments, the current was evoked within DRN by a bipolar tungsten electrode. The signals were filtered at 2 kHz, digitized at 5 kHz sampling rate, and stored in a computer (Digidata; Axon Instrument). The data was analyzed by Minianlysis software (Synaptosoft Inc.).

Radioimmunoassay of AVP—To determine the basal levels of CSF AVP hormones, individually housed male mice were left undisturbed for 4 weeks and anesthetized with Avertin® in the light phase. Collection of CSF samples was based on what was described by DeMattos *et al.* (26). Clear CSF samples were drawn (10–15 μl/animal) from the cisterna magna using a glass capillary to avoid blood contamination of CSF. All of the samples were centrifuged for at 3000 × *g* for 15 min at 4 °C. The

clear supernatants were stored at −70 °C until the assay. The AVP levels were measured in 10-μl samples of CSF using a radioimmunoassay kit (Peninsula Laboratories) according to the manufacturer's protocols.

Ventricular Infusion of a V1a Receptor Antagonist—Male mice were anesthetized with Avertin®, and an osmotic pump (flow rate, 0.25 μl/h; ALZET) for infusion of AVP antagonist was stereotaxically placed with its tip into a lateral ventricle region. The stereotaxic coordinates for the microinfusion were anterior-posterior = −0.22 mm from the bregma; lateral = ±1.0 mm; ventral = −2.8 mm. The animals were infused with either vehicle (phosphate-buffered saline) or the selective V1a receptor antagonist $d((CH_2)_5^1,Tyr(Me)^2,Arg^8)$-vasopressin (AVP-8, 10 ng/μl; Bachem) into the lateral ventricle using the pump according to the manufacturer's protocols. After 10 days, the resident-intruder test was performed.

Analysis of Monoamines by High Pressure Liquid Chromatography (HPLC)—The adult brain was rapidly removed and dissected on ice as previously described (27). The hypothalamus was immediately homogenized in 0.1 M perchloric acid with a Tissue Tearer (Biospec) at 30,000 rpm for 30 s. The homogenates (5% w/v) were centrifuged at 48,000 × *g* for 20 min at 4 °C, and the supernatants were collected and stored at −70 °C until analysis. The endogenous levels of norepinephrine, dopamine, 5-HT, and their metabolites 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindolacetic acid (5-HIAA) were determined by reverse phase HPLC with an ICA-3063 electrochemical detector (Toa). Briefly, 10-μl portions of the samples were separated using a C18 reversed-phase column (octadecyl silica; 4.6 mm × 150 mm; NaKalai, Japan) and separated using a mobile phase of 85:15 0.5 M KH_2PO_4, pH 3.2, containing 2.5 mM 1-octane sulfate and 10-μM EDTA/methanol delivered at 0.9 ml/min. The oxidation potential of the detector was fixed at 750 mV using a glass carbon working electrode *versus* an Ag/AgCl reference electrode. The peak areas of the internal standard (dihydrobenzylamine) were used to quantify the sample peaks. The values obtained were expressed as μg/g of tissue wet weight.

Statistical Analyses—All of the values are presented as the means ± S.E. of the means (S.E.), and statistical analysis was performed using Student's *t* test. The values were considered statistically different at $p < 0.05$.

RESULTS

Enhanced Aggression in $Ca_v2.2^{-/-}$ Mice—To quantify the aggressive behaviors of $Ca_v2.2^{-/-}$ male mice, we performed the resident-intruder test (21). As shown in Fig. 1 (*A* and *B*), $Ca_v2.2^{-/-}$ mice showed a significantly shortened latency to the first attack on the intruder mouse and an increased number of attacks compared with wild type male littermates, showing that $Ca_v2.2^{-/-}$ mice are significantly more aggressive than the wild type mice. Because dominant males are more aggressive than subordinate males in intermale conflicts to maintain their social position (28), we next performed the water competition test (22). As shown in Fig. 1*C*, $Ca_v2.2^{-/-}$ mice displayed a longer duration of water consumption than the wild type. There was no significant difference in the duration of water consumption over a 2-min test period between the two genotypes, when they were separately housed (data not shown). These results demonstrate that $Ca_v2.2^{-/-}$ mice are dominant over the wild type mice in social interactions.

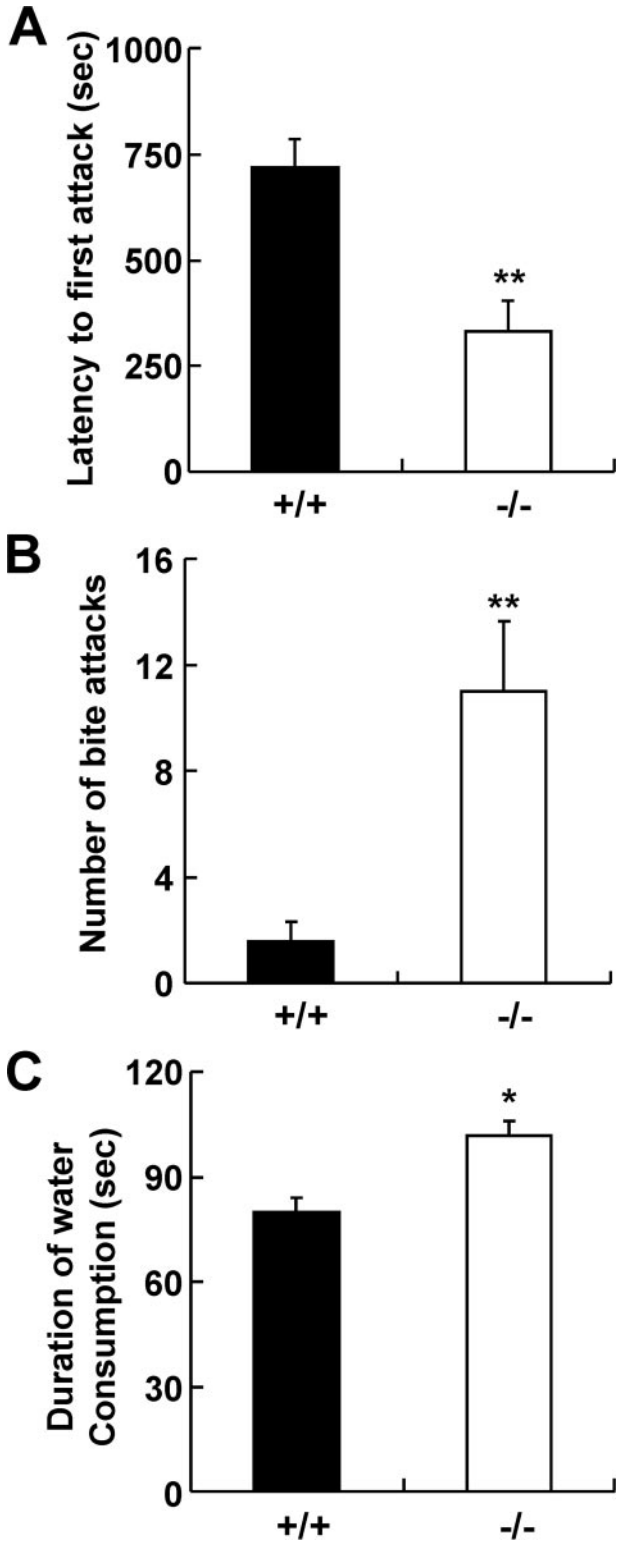

FIGURE 1. **Enhanced aggressive behaviors in $Ca_v2.2^{-/-}$ male mice.** In the resident-intruder test, $Ca_v2.2^{-/-}$ mice ($n = 12$) showed a significantly shorter latency to the first attack (*A*) and an increased number of bite attacks (*B*) to an intruder than wild type littermates ($n = 11$). *C*, in the water competition test, $Ca_v2.2^{-/-}$ mice showed a longer duration of water consumption than the wild type. *, $p < 0.05$; **, $p < 0.01$.

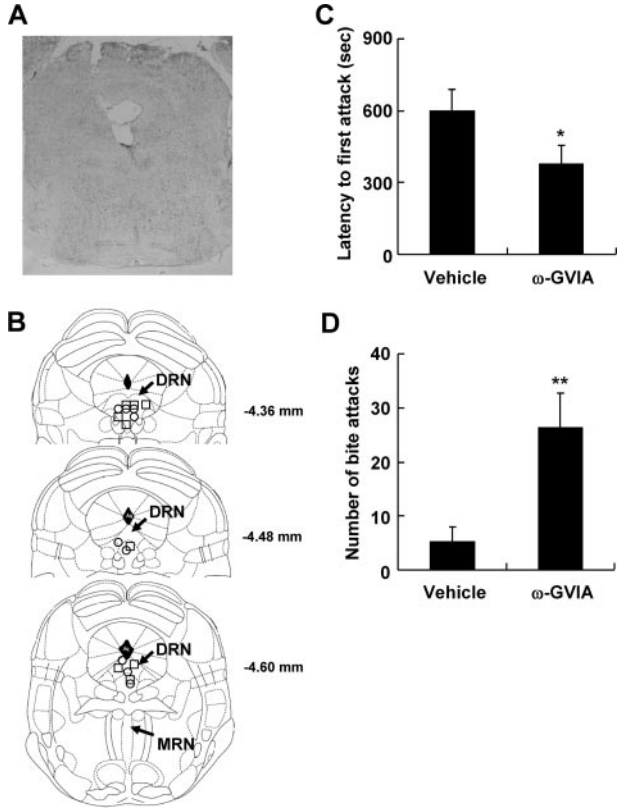

FIGURE 2. **Escalated aggression in ω-conotoxin GVIA (ω-GVIA) intra-raphe-injected wild type male mice.** *A*, representative photomicrograph of a mouse coronal brain section that was stained with cresyl violet to visualize the cannula tract and injection site (×2) in DRN. *B*, location of microinjection sites in the DRN. Schematic representations of mouse coronal brain sections based on the stereotaxic atlas of Paxinos and Franklin (24). The values indicate the distance (mm) of the section from bregma. The *open circles* represent the sites where ω-GVIA ($n = 9$) were injected, and the *open squares* show the sites where vehicle ($n = 9$) were injected. *MRN*, median raphe nucleus. The ω-GVIA-injected mice ($n = 9$) showed a shorter latency to the first attack (*C*) and an increased number of bite attacks (*D*) to an intruder in the resident-intruder test compared with the vehicle-injected control mice ($n = 9$). *, $p < 0.05$; **, $p < 0.01$.

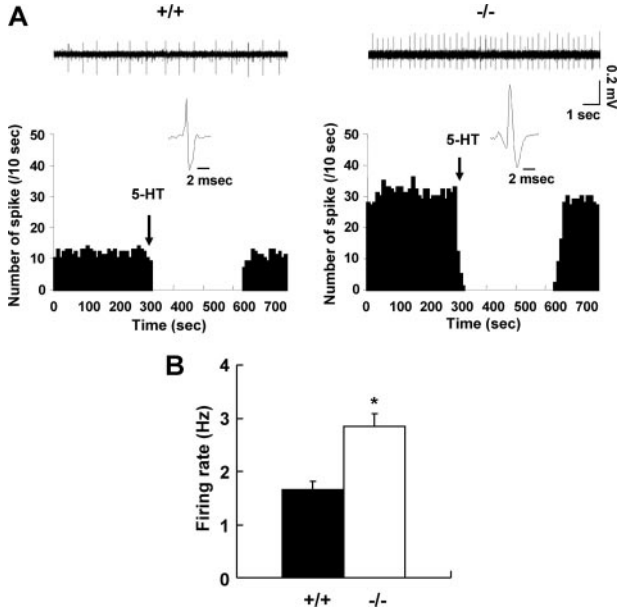

FIGURE 3. **Single-unit activity of 5-HT neurons in the DRN.** *A*, examples of the single-unit activity in a DRN. Increased firing activity was observed in $Ca_v2.2^{-/-}$. *B*, there was a significant difference in the firing rate (wild type, $n = 14$; $Ca_v2.2^{-/-}$, $n = 16$). *, $p < 0.01$.

Intra-raphe Injection of an N-type Ca^{2+} Channel Blocker—To examine whether the inactivation of N-type Ca^{2+} channels in the DRN is responsible for the increased aggression of $Ca_v2.2^{-/-}$ mice, we focally injected an N-type Ca^{2+} channel blocker, ω-conotoxin GVIA (ω-GVIA), into the DRN of wild type male mice according to the procedures described under "Experimental Procedures." The mice microinjected with ω-GVIA showed symptoms mimicking the phenotype of the $Ca_v2.2^{-/-}$ mice: a significantly reduced latency to the first attack and an increased number of bite attacks on the intruder compared with the saline-treated control mice (Fig. 2, *C* and *D*). Histological analyses confirmed that injection placements were correctly located in the dorsal raphe (Fig. 2*B*). An example of the cannular tract and injection site is shown in Fig. 2*A*. These results indicate that the highly aggressive behaviors of $Ca_v2.2^{-/-}$ mice was primarily due to a lack of N-type Ca^{2+} channels in the DRN and also suggest that a developmental anomaly is not a factor in the expression of the aggression phenotype.

Increased Activities of 5-HT Neurons in $Ca_v2.2^{-/-}$ Mice—To define the alterations in the DRN that resulted from the mutation and were responsible for the aggression phenotype, we examined the physiological properties of the DRN in slices. First, we recorded extracellularly the single-unit activities from the putative 5-HT neurons, identified according to the criteria described in literature (15, 17), in the DRN slices of the wild type and $Ca_v2.2^{-/-}$ mice. Firing was induced by adding the α_1-adrenoreceptor agonist phenylephrine (3 μM). The results show that 5-HT neurons of the $Ca_v2.2^{-/-}$ DRN fired more frequently than those of the wild type mice (wild type, 1.66 ± 0.16 Hz; $Ca_v2.2^{-/-}$ mice, 2.85 ± 0.23 Hz; $p < 0.01$; Student's *t* test) (Fig. 3, *A* and *B*).

For further analysis of 5-HT neurons in the DRN of the $Ca_v2.2^{-/-}$ mice, we next tried to identify 5-HT neurons, based on electrophysiological criteria as described under "Experimental Procedures" and supplemental data and measured their activity by whole cell patch clamp recordings with the procedures. We analyzed individual action potential shapes in the current clamp recordings. There was no difference in the amplitude or duration of action potential or in the amplitude of after hyperpolarization between the wild type and $Ca_v2.2^{-/-}$ mice (supplemental Fig. S1 and Table S1). However, when we slightly depolarized membrane potential of the cells by an intracellular current injection of +20 pA, we consistently observed that the 5-HT neurons in the $Ca_v2.2^{-/-}$ DRN fired more frequently than those of the wild type mice (wild type, 2.48 ± 0.27 Hz; $Ca_v2.2^{-/-}$, 3.57 ± 0.36 Hz; $p < 0.05$; Student's *t* test) (Fig. 4, *A–C*, before bicuculline). These results were consistent with the extracellular single-unit recordings as described above (Fig. 3).

The activity of 5-HT neurons can be highly modulated by input from the GABAergic interneurons (19). To test whether the increased firing rate of 5-HT neurons in the $Ca_v2.2^{-/-}$ DRN was due to a decreased GABA inhibition, we recorded neuronal activities from identified 5-HT neurons, based on electrophysi-

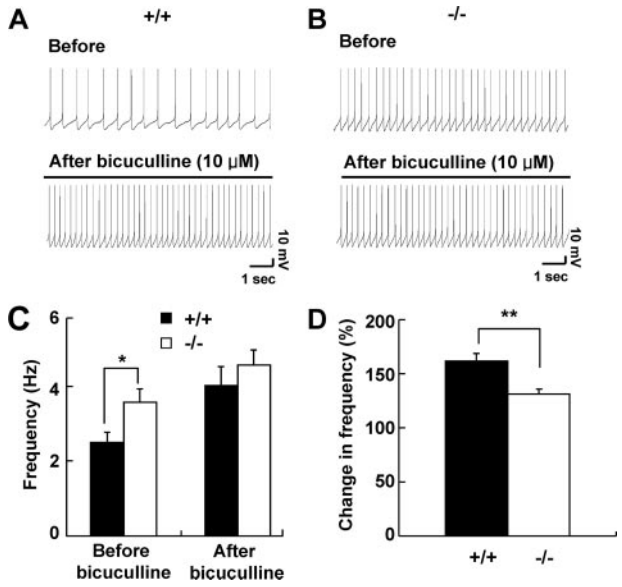

FIGURE 4. **Firing of 5-HT neurons in the absence of inhibitory transmissions.** Sample traces of firing in 5-HT neurons of the wild type (*A*) and $Ca_v2.2^{-/-}$ (*B*). Firings were induced by the intracellular current injection (+20 pA) with or without bicuculline (10 μM). *C*, before the application of bicuculline, there was a significant difference in firing frequency between wild type ($n = 10$) and $Ca_v2.2^{-/-}$ ($n = 14$). After the treatment of bicuculline, the firing frequency increased in both genotypes, reaching to a similar level. However, there was a significant difference in the percentage of change of firing frequency after an application of bicuculline (*D*). *, $p < 0.05$; **, $p < 0.01$.

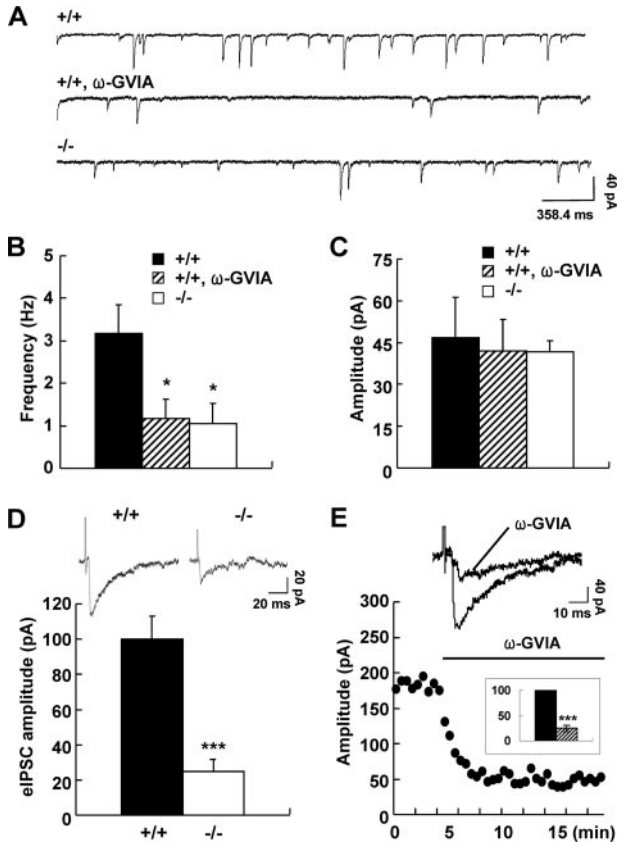

FIGURE 5. **sIPSC and eIPSC of 5-HT neurons in the DRN.** *A*, sample traces of sIPSC in the wild type and $Ca_v2.2^{-/-}$ slices. *B* and *C*, the frequency of sIPSC was reduced in $Ca_v2.2^{-/-}$ (wild type, $n = 5$; $Ca_v2.2^{-/-}$, $n = 5$) without changes in the amplitude. In the presence of ω-GVIA ($n = 5$), the frequency of sIPSC of the wild type was reduced to a level similar to that of $Ca_v2.2^{-/-}$, but no difference was observed in the amplitude. *D*, the peak amplitude of eIPSC was drastically reduced in $Ca_v2.2^{-/-}$ (wild type, $n = 5$; $Ca_v2.2^{-/-}$, $n = 5$). *E*, in the presence of ω-GVIA ($n = 5$), the peak amplitude of eIPSC in the wild type was reduced by ~75%, compared with that of the vehicle control (*inset*). *, $p < 0.05$; ***, $p < 0.0001$.

ological criteria, in the presence of bicuculline, a $GABA_A$ receptor antagonist. The application of bicuculline (10 μM) increased the firing rates in both $Ca_v2.2^{-/-}$ and the wild type to a similar level (the wild type with bicuculline, 4.01 ± 0.51 Hz; $Ca_v2.2^{-/-}$ with bicuculline, 4.56 ± 0.39 Hz; $p = 0.48$; Student's *t* test) (Fig. 4*C*). However, the magnitude of the increase of the firing rate was significantly greater ($p < 0.01$; Student's *t* test) in the wild type (161.56 ± 7.55%) than in the $Ca_v2.2^{-/-}$ (131.10 ± 4.75%), reflecting the already increased level of firing in the mutant, presumably because of decreased inhibition (Fig. 4*D*). These results suggested a possibility that the increased firing rate in the 5-HT neurons of $Ca_v2.2^{-/-}$ mice was due to an impaired GABA transmission in the DRN.

Decreased GABA Release in the $Ca_v2.2^{-/-}$ DRN—To define further how the inhibitory transmission is altered in the $Ca_v2.2^{-/-}$ DRN, we measured spontaneous and evoked inhibitory postsynaptic currents (sIPSC and eIPSC, respectively) from identified 5-HT neurons, based on electrophysiological criteria, in DRN slices under a voltage-clamp configuration. We found that the frequency of sIPSC in $Ca_v2.2^{-/-}$ neurons was significantly reduced compared with that of wild type neurons (wild type, 3.17 ± 0.68 Hz; $Ca_v2.2^{-/-}$, 1.05 ± 0.48 Hz; $p < 0.05$; Fig. 5*B*), without any significant change in the amplitude (wild type, 46.84 ± 14.42 pA; $Ca_v2.2^{-/-}$, 41.74 ± 3.94 pA; $p = 0.85$; Fig. 5*C*). Furthermore, this reduction of sIPSC in the $Ca_v2.2^{-/-}$ was mimicked in the wild type slices by an N-type channel blocker; when ω-GVIA (1 μM) was added to the recording solution of the wild type slice ($n = 5$), the frequency of the sIPSC was reduced to the values displayed in the $Ca_v2.2^{-/-}$ (wild type with ω-GVIA, 1.18 ± 0.45 Hz; Fig. 5*B*), without any change in the amplitude (wild type with ω-GVIA, 42.04 ± 11.40 pA; Fig. 5*C*).

Next, we recorded the eIPSC in 5-HT neurons following a focal stimulation within the DRN. The maximum amplitude of eIPSC in the $Ca_v2.2^{-/-}$ was lower than that in the wild type (wild type, 172.89 ± 20.53 pA; $Ca_v2.2^{-/-}$, 32.69 ± 5.92 pA; $p < 0.0001$; Fig. 5*D*). Furthermore, application of ω-GVIA to the wild type slice significantly reduced the amplitude of eIPSC, by 75% (Fig. 5*E*). These results on sIPSC and eIPSC indicate that presynaptic GABA release is impaired with normal postsynaptic GABA responses in the $Ca_v2.2^{-/-}$ DRN and suggest that N-type Ca^{2+} channels have a crucial role in the release of GABA in the DRN.

In contrast to IPSC, there was no difference in the frequency or amplitude of the spontaneous excitatory postsynaptic current (sEPSC) between the two genotypes (supplemental Fig. S2, *B* and *C*). The maximum amplitude of evoked excitatory postsynaptic current (eEPSC) in the $Ca_v2.2^{-/-}$ was also similar to that of the wild type (supplemental Fig. S2, *D* and *E*). Thus, these results indicate that excitatory transmissions into 5-HT neurons were not altered in the $Ca_v2.2^{-/-}$ DRN.

Increased Levels of CSF AVP and Hypothalamic 5-HT/5-HIAA in $Ca_v2.2^{-/-}$—Central AVP is known to play an important role in the regulation of aggressive behaviors. In particular,

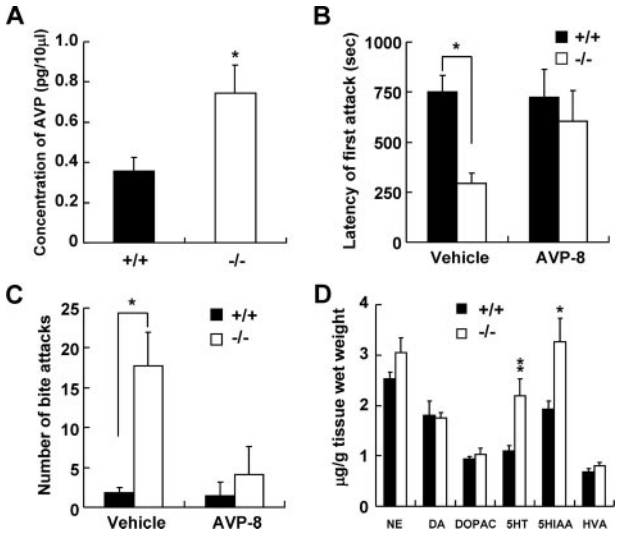

FIGURE 6. **Increased amount of CSF AVP and hypothalamic 5-HT/5-HIAA in the $Ca_v2.2^{-/-}$ mice.** *A*, $Ca_v2.2^{-/-}$ mice showed a significantly higher concentration of CSF AVP (wild type, $n = 12$; $Ca_v2.2^{-/-}$, $n = 15$) than wild type. Following administration of the V1a receptor antagonist, $d((CH_2)_5^1,Tyr(Me)^2,Arg^8)$-vasopressin (AVP-8), the latency to the first attack (*B*) and the number of bite attacks (*C*) of $Ca_v2.2^{-/-}$ mice ($n = 8$) became similar to those of the wild type mice ($n = 8$). However, vehicle infusion did not affect the enhanced aggressiveness of the $Ca_v2.2^{-/-}$ mice ($n = 7$) over the wild type mice ($n = 7$). *D*, HPLC measurements of the concentrations of monoamines and their metabolites in the hypothalamus of the animals. The levels of 5-HT and the 5-HIAA of $Ca_v2.2^{-/-}$ mice were significantly higher in $Ca_v2.2^{-/-}$ hypothalamus ($n = 7$) than the wild type ($n = 9$). There was no significant difference in concentrations of other monoamines or their metabolites between $Ca_v2.2^{-/-}$ mice and wild type littermates. *, $p < 0.05$; **, $p < 0.01$.

an increased level of CSF AVP has been consistently implicated in enhanced aggressive behaviors in various species including humans (29–31). We thus tried to measure AVP levels in the CSF of the $Ca_v2.2^{-/-}$ mice and found that the $Ca_v2.2^{-/-}$ mice have a significantly higher level of AVP in the CSF compared with wild type mice (Fig. 6*A*). We then examined whether the increased level of AVP is related to the enhanced aggression of $Ca_v2.2^{-/-}$ mice. It has been demonstrated that microinjection of the AVP receptor (V1a) antagonist $d(CH_2)_5$,Tyr(Me)AVP into the anterior hypothalamus of the golden hamster rapidly inhibits aggression (29, 32). In our experiments, microinfusion of an AVP receptor V1a antagonist, $d((CH_2)_5^1,Tyr(Me)^2,Arg^8)$-vasopressin (AVP-8, 10 ng/μl, 0.25 μl/hr), into the lateral ventricle using an osmotic pump reduced the level of aggressiveness of $Ca_v2.2^{-/-}$ mice (Fig. 6, *B* and *C*). However, the same dose of the antagonist did not affect the aggression behavior of wild type mice (Fig. 6, *B* and *C*). These results suggest that the elevation of AVP in the CSF is at least in part involved in the enhanced aggressive behaviors of $Ca_v2.2^{-/-}$ mice.

A large number of studies have revealed that AVP is produced in the hypothalamus, including the paraventricular nucleus, supraoptic nucleus, anterior hypothalamus, and suprachiasmatic nucleus, in addition to other brain areas including bed nucleus of the stria terminalis, medial amygdale, and lateral septum (32–35). Moreover, those AVP neurons in the hypothalamus are innervated by serotonergic neurons originating in the dorsal and median raphe nucleus (36, 37). Therefore, we next attempted to measure the amount of monoamines including 5-HT in the hypothalamus of the $Ca_v2.2^{-/-}$ mice using HPLC. The results revealed that the levels of 5-HT and 5-HIAA were significantly elevated in the $Ca_v2.2^{-/-}$ hypothalamus compared with those of the wild type (Fig. 6*D*), indicating increased 5-HT release in the $Ca_v2.2^{-/-}$ hypothalamus. However, there was no significant difference in the levels of other monoamines between the two genotypes.

DISCUSSION

In this study, we examined aggressive behaviors in the $Ca_v2.2^{-/-}$ mice and tried to elucidate the neural mechanisms underlying the enhanced aggression phenotype of the $Ca_v2.2^{-/-}$ mice. $Ca_v2.2^{-/-}$ mice showed impairment in the inhibitory transmission in the DRN, which led to disinhibition of the 5-HT neuron, resulting in increased activity of the 5-HT neurons. In addition, $Ca_v2.2^{-/-}$ showed significantly increased AVP secretion in the brain, together with an increase of 5-HT in the hypothalamus. These results suggest the possibility that N-type Ca^{2+} channels are involved in the suppression of aggression through their function in the GABAergic synaptic transmission in the DRN.

GABA neurotransmission is one of the major contributors to the control of the firing activity of 5-HT neurons in the DRN together with 5-HT_{1A} autoreceptor and noradrenergic input (17, 19). Because the basal spontaneous firing rates of 5-HT neurons were not altered in 5-HT_{1A} receptor knock-out mice (38) and an exogenous 5-HT application inhibited the firing activity of 5-HT neurons of the $Ca_v2.2^{-/-}$ DRN (Fig. 3*A*), the increased firing activity of 5-HT neurons in the $Ca_v2.2^{-/-}$ is not likely to be caused by an impairment in the 5-HT autoreceptor-mediated inhibition in the $Ca_v2.2^{-/-}$ DRN. In addition, the noradrenergic system does not appear to have been affected in $Ca_v2.2^{-/-}$ mice, because there was no significant difference between $Ca_v2.2^{-/-}$ and wild type mice in the level of norepinephrine in the plasma in previous studies (3) or in the hypothalamus in this report (Fig. 6*D*). Therefore, the increased firing activity of the 5-HT neurons, based on electrophysiological criteria, in the $Ca_v2.2^{-/-}$ DRN is likely due to a failure in the GABAergic system, specifically, an impairment of the presynaptic GABA release as shown in Figs. 4 and 5. In fact, it has been shown that the $Ca_v2.2$ is highly expressed in the DRN of adult rats (20). Thus, the $Ca_v2.2$ could be localized at GABAergic presynaptic terminals in the DRN, as was shown in the cortical neurons (39). Co-localization studies of $Ca_v2.2$ and GABAergic neuron markers in the DRN would be needed to confirm this relationship.

Numerous studies have demonstrated that the central 5-HT system is strongly linked to aggressive behaviors. However, there are conflicting reports on the correlation between the level of 5-HT in CSF and aggression. In many reports, reduced CSF concentration of the 5-HT metabolite, 5-HIAA, is implicated in severe forms of aggression, such as impulsive aggression or violence, in humans and other primates (40, 41), which has been supported by pharmacological or genetic studies in animals (42, 43). In contrast, a positive correlation was found between the level of trait-like aggression (high or low) and levels of 5-HT and 5-HIAA in rats (44). Besides, a number of studies have reported that 5-HT release is stimulated by aggressive social interaction in lizards (45–47) and is increased during

performance of aggressive behaviors in rats (13), suggesting that the enhanced 5-HT release is an important event for promoting and sustaining aggressive behavior. In line with this hypothesis, in recent reports 5-HT_{1A} and 5-HT_{1B} agonists, which reduce extracellular concentration of 5-HT through their inhibitory actions at somatodendritic and terminal autoreceptors (48, 49), decreased offensive aggressive behavior in rats (50, 51). Furthermore, in mice deficient for monoamine oxidase A, increased levels of 5-HT in whole brains caused a markedly enhanced aggressiveness (52), a phenotype also observed in humans with the same mutation (53). Interestingly, CSF, plasma, and urinary levels of monoamine metabolites including 5-HIAA were positively correlated with aggression and impulsivity/hyperactivity in ADHD boys (9, 54). Moreover, recent studies have shown that rapid depletion of tryptophan, 5-HT precursor, in ADHD patients reduced aggression, suggesting that enhanced serotonergic activity caused reactive aggressive behaviors in ADHD (55).

Although our results are consistent with the cases of a positive correlation between an increase of 5-HT and aggression, an explanation for the discrepancy between the two conflicting groups of observations is not available at the moment. From our findings and previous data, however, we may suggest that chronic and sustained enhancement of 5-HT release is positively associated with both normal offensive aggression (for maintaining territory or social dominance) (28, 51) and pathophysiological aggression characterized by psychiatric patients, such as ADHD, whereas a reduced central 5-HT activity may be more specifically implicated in abnormal forms of aggression (*i.e.* "irritable" impulsive aggression) but not in nonviolent aggression such as social dominance or competitiveness (41, 56).

Physiological responses to stressful social interaction are highly conserved in vertebrates and include the secretion of stress hormones such as AVP (57). Our results are consistent with previous studies showing that elevated AVP in the brain has been consistently implicated in enhanced aggressive behaviors in various species including humans (29–31). Moreover, administration of the AVP receptor V1a antagonist reduced the aggressive behavior of the $Ca_v2.2^{-/-}$ mice.

AVP-secreting neurons in the hypothalamus are also known to associate with 5-HT system. Previous studies have shown that the AVP neurons in paraventricular nucleus, supraoptic nucleus, and anterior hypothalamus are innervated by 5-HT nerve fibers from the DRN (32, 36, 37), which is involved in the regulation of aggression in male or female rodents (32, 58, 59). Moreover, an intracerebroventricular infusion of 5-HT induced an extracellular vasopressin release in the paraventricular nucleus, suggesting a direct effect of 5-HT on vasopressin synthesis and central release (60). In monoamine oxidase A-deficient mice, the increased level of 5-HT in the brain led to not only high aggression (52) but also to an activation of AVP expression in the paraventricular nucleus and supraoptic nucleus (61). Thus, increased 5-HT neuronal activity in the $Ca_v2.2^{-/-}$ DRN might give rise to an elevated AVP secretion, which may be responsible for the hyperaggression in the $Ca_v2.2^{-/-}$ mice. However, we cannot rule out the possibility that secretion of AVP is also increased from other brain regions, because AVP is released from other brain areas (33–35).

In conclusion, our results suggest that the role of N-type Ca^{2+} channels in GABA transmission in the DRN is required for the suppression of aggression. The deletion of this N-type channel function led to increased firing activity of 5-HT neurons in the DRN and an elevated level of AVP in the brain, resulting in an enhanced aggression in the $Ca_v2.2^{-/-}$ mice. These results may provide us with a new insight into the neurobiology underlying aggressive behaviors in animals and humans.

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