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Presynaptic GABA_B Receptors Decrease Neurotransmitter Release in Vestibular Nuclei Neurons During Vestibular Compensation

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Abstract

Unilateral damage to the peripheral vestibular receptors precipitates a debilitating syndrome of oculomotor and balance deficits at rest, which extensively normalize during the first week after the lesion due to vestibular compensation. In vivo studies suggest that GABA_B receptor activation facilitates recovery. However, the presynaptic or postsynaptic sites of action of GABA_B receptors in vestibular nuclei neurons after lesions have not been determined. Accordingly, here presynaptic and postsynaptic GABA_B receptor activity in principal cells of the tangential nucleus, a major avian vestibular nucleus, was investigated using patch-clamp recordings correlated with immunolabeling and confocal imaging of the GABA_B receptor subunit-2 (GABA_BR2) in controls and operated chickens shortly after unilateral vestibular ganglionectomy (UVG). Baclofen, a GABA_B agonist, generated no postsynaptic currents in principal cells in controls, which correlated with weak GABA_BR2 immunolabeling on principal cell surfaces. However, baclofen decreased miniature excitatory (mEPSC) and GABAergic inhibitory (mIPSC) events in principal cells in controls, compensating and uncompensated chickens three days after UVG, indicating the presence of functional GABA_B receptors on presynaptic terminals. Baclofen decreased GABAergic mIPSC frequency to the greatest extent in principal cells on the intact side of compensating chickens, with concurrent increases in GABA_BR2 pixel brightness and percentage overlap in synaptotagmin2 (Syt2)-labeled terminals. In uncompensated chickens, baclofen decreased mEPSC frequency to the greatest extent in principal cells on the intact side, with concurrent increases in GABA_BR2 pixel brightness and percentage overlap in Syt1-labeled terminals. Altogether, these results revealed changes in presynaptic GABA_B receptor function and expression which differed in compensating and uncompensated chickens shortly after UVG. This work supports an important role for GABA_B autoreceptor-mediated inhibition in vestibular nuclei neurons on the intact side during early stages of vestibular compensation, and a role for GABA_B

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heteroreceptor-mediated inhibition of glutamatergic terminals on the intact side in the failure to recover function.

Keywords

vestibular deafferentation; synaptic plasticity; presynaptic receptors; excitation; inhibition

Gamma-amino-butyric acid (GABA) is the main inhibitory neurotransmitter in the adult brain with multiple important and diverse functions during development (Ben-Ari, 2002; Owens and Kriegstein, 2002; Lujan et al., 2005) and after lesions in sensory systems (Hendry and Jones, 1986; 1988; Akhtar and Land, 1991; Rosier et al., 1995; Fuchs and Salazar, 1998; Moore et al., 2002) (for review see, Caspary et al., 2008). The two major types of GABA receptors, GABA_A and GABA_B, are found on presynaptic and postsynaptic membranes. GABA_A receptors function mainly as ligand-gated chloride channels generating fast synaptic inhibition, while GABA_B receptors are G-protein coupled metabotropic receptors which produce slow, prolonged inhibition. Presynaptic GABA_B receptors form two functional subtypes, GABA_B autoreceptors on GABAergic terminals and GABA_B heteroreceptors positioned on non-GABAergic terminals (for review, see Bettler and Tiao, 2006).

The vestibular nuclei are composed of a diverse assortment of second-order vestibular neurons, with some of them functioning as reflex projection neurons transmitting signals from the peripheral vestibular sensory epithelium to motor neurons in the vestibular reflex pathways. In a wide range of species, GABA-mediated inhibition plays a critical role in regulating signal processing in the vestibular nuclei, since these neurons receive massive GABAergic inputs from the cerebellum, contralateral vestibular nuclei, inferior olivary nucleus and local interneurons (de Waele et al., 1995; Highstein and Holstein, 2006). Both GABA_A and presynaptic and postsynaptic GABA_B receptors are expressed in the mammalian vestibular nuclei (Holstein et al., 1992; Gallagher et al., 1992; Vibert et al., 1995; Eleore et al., 2005; Gliddon et al., 2005; Heskin-Sweezie et al., 2010). The tangential nucleus represents a major avian vestibular nucleus, composed primarily of the principal cells (80%), which are distinguished by large oval, glutamatergic cell bodies aligned in rows between the primary vestibular fibers near the lateral surface of the medulla oblongata (Peusner and Morest, 1977; Popratiloff and Peusner, 2011). The neurotransmitter phenotypes of the elongate (19%) and giant cells (1%) in the tangential nucleus have not been identified. Principal cells receive multiple inputs from diverse sources, including excitatory input from the canals, otoliths and high cervical spinal cord (C1-C2) (Gross, 1985; Cox and Peusner, 1990; Popratiloff et al., 2004; Sato et al., 1997). Although no local GABAergic or glycinergic interneurons are found in the tangential nucleus (Popratiloff and Peusner, 2011), the principal cells receive GABAergic inputs from the cerebellar flocculus. The neurotransmitter phenotype of inputs from the contralateral medial vestibular nucleus (MVN) (Cox and Peusner, 1990) has not been established. All the principal cells are vestibular reflex projection neurons whose axons project to the oculomotor nuclei and/or cervical spinal cord (Wold, 1978; Cox and Peusner, 1990; Gottesman-Davis and Peusner, 2010). Thus, the tangential nucleus differs from most vestibular nuclei in that their major

output neurons have a distinctive morphology and all of them function as projection neurons within the vestibular reflex pathways. Previous amino acid immunolabeling studies reveal that GABAergic terminals compose about half of the synaptotagmin-1 (Syt1)-labeled terminals in the tangential nucleus (Popratiloff and Peusner, 2011). In voltage-clamp recordings, both GABA_A and GABA_B receptor-mediated currents are generated in principal cells from brain slices of 16-day-old chick embryos (E16) (Shao et al., 2003).

Unilateral peripheral vestibular lesions precipitate a complex syndrome of oculomotor and balance deficits (patients: Badke et al., 2002; chicken: Aldrich and Peusner, 2002; Shao et al., 2009; 2012) due to dysfunction of the vestibular sensorimotor reflex pathways (Llinas and Walton, 1979). In different species, the static symptoms usually normalize extensively during the first week after the lesion due to a process called vestibular compensation (Smith and Curthoys, 1989; Halmagyi et al., 2010). Immediately after unilateral vestibular ganglionectomy (UVG), four-day-old hatchling (H4) chickens show severe static symptoms, which generally start to recover by three days (Aldrich and Peusner, 2002). However, a significant portion of the operated chickens fails to recover by three days, the uncompensated chickens (47-48%; Shao et al., 2009; 2012). Presently, vestibular compensation is thought to involve functional reorganization of the synaptic connections in broadly distributed regions of the central nervous system which process vestibular signals, including the vestibular nuclei, cerebellum, inferior olivary nucleus, spinal cord, and visual system (Llinas and Walton, 1979; for review, see Dieringer, 1995). Research has focused on the vestibular nuclei because they must be intact for recovery to occur (Spiegel and Demetriades, 1925; Precht et al., 1966). While the exact cellular mechanisms underlying vestibular compensation are uncertain, it is thought that recovery from the static symptoms involves rebalancing the overall excitability in vestibular nuclei neurons bilaterally (for review, see Beraneck and Idoux, 2012; Lambert and Straka, 2012; Peusner et al., 2012). Major changes in the frequency and kinetics of glutamatergic and GABAergic spontaneous synaptic events occur in the vestibular nuclei neurons after peripheral vestibular lesions, suggesting that both presynaptic and postsynaptic factors participate in the recovery (Shao et al., 2012). Collectively, these properties must regulate rebalancing of neuronal excitability. However, the factors leading to changes in frequency and kinetics of spontaneous synaptic events are uncertain.

In general, behavioral studies support an important role for GABA_B receptors in vestibular compensation, since systemic injection of the specific GABA_B agonist, baclofen, decreases spontaneous nystagmus in rats after unilateral labyrinthectomy (UL) (Magnusson et al., 2000; 2002) and accelerates recovery of the postural reflexes in mice (Heskin-Sweezie et al., 2010). At present, the effect of GABA_B receptors on specific excitatory and inhibitory inputs contacting vestibular reflex projection neurons has not been investigated after lesions. Accordingly, here the presynaptic or postsynaptic action of GABA_B receptors in principal cells of the chick tangential nucleus were studied and distinguished unambiguously before and after UVG by correlating patch-clamp recordings with immunolabeling and high resolution confocal imaging of these receptors.

Experimental Procedures

Experimental animals

Cold, freshly fertilized, white Leghorn chick eggs (*Gallus gallus*) were purchased from CBT Farms (Chestertown, MD) or Charles River Spafas (North Franklin, CT) and maintained until hatching in an egg incubator equipped with circulating air, egg rotation unit, and temperature and humidity controls (model 1502, G.Q.F. Manufacturing Co., Savannah, GA). Twenty-four hours after hatching, chickens were identified as one day-old hatchlings (H1). The chickens were housed in plastic cages equipped with heating lamps at the University's Animal Research Facility. The experiments were performed in accordance with protocols approved by the Institution Animal Care and Use Committee of the George Washington University. The experiments also conformed to the guidelines of the National Research Council (2003) for the care and use of animals in research. Considerable care was taken to minimize the number of animals used in the experiments and their suffering.

These structure/function studies were performed on a total of 112 chickens. The electrophysiological experiments were undertaken on 97 chickens, including controls (n=18), operated chickens one day after UVG (n=23), compensating (n=30) and uncompensated (n=26) chickens three days after UVG. The immunolabeling experiments were performed on 15 animals, including controls (n=7), compensating (n=4) and uncompensated (n=4) chickens three days after UVG. Sham-operated animals were not prepared because no difference was detected between sham-operated animals and controls in a previous study of Kv1.1 and Kv1.2 immunolabeling in hatchling chickens, suggesting that anesthesia and stress of surgery produced no measurable effects on these animals (Shao et al., 2009).

Unilateral vestibular ganglionectomy (UVG)

UVG was performed on H4 chickens which were sacrificed one (H5) or three days (H7) later. Under general anesthesia (ketamine, 100 mg/kg, Fort Dodge Animal Health, Fort Dodge, IO; xylazine, 20 mg/kg; Akorn Inc., Decatur, IL), the left vestibular ganglion was ablated completely, as previously described (Aldrich and Peusner 2002; Pollack et al., 2004; Shao et al. 2009; 2012). On regaining consciousness three to four hours after surgery, the operated chickens received daily subcutaneous injections of sterile lactated Ringer's solution (Braun Medical, Inc., Irvine, CA) to replace lost fluid (1 ml Ringer's solution/1 gram body weight loss) (National Research Council, 2003). One day after UVG, the operated chickens received an intramuscular injection of 0.1 ml penicillin-G prophylactically (Bimeda Inc., Le Sueur, MN) and starting on the second day, all the operated chickens received Clavamox antibiotic via gavage (Pfizer, New York, NY)(62.5 mg/ml, 1.5 ml/day).

After sacrificing the operated animals, the cranium and brainstem were inspected visually for the complete removal of the vestibular ganglion on the lesion side and absence of gross brain damage, edema, and hemorrhage in the brain. No brain damage was detected in the compensating or uncompensated chickens. No signs of principal cell death were observed on the lesion side of the compensating chickens up to 56 days after UVG (Aldrich and Peusner, 2002), supporting the idea that the principal cells do not undergo a critical developmental

period at H4 or thereafter which might make them more susceptible to cell death after vestibular deafferentation (Vale and Sanes, 2002).

Brain slice preparation and solutions

Chickens were decapitated and the part of the cranium containing the brainstem was dissected out as previously described (Shao et al., 2003, 2004). The brainstem was glued (Cyanoacrylic glue; Crazy Glue, Elmer's Products Inc., Columbus, OH) to a vibratome chuck, which was immersed in sucrose ACSF (1–2°C) contained in a vibratome bath (VT1000S; Leica Instruments, Bannockburn, IL). Transverse brainstem slices containing the tangential nucleus were cut at 240 μm using a sapphire knife (Delaware Diamond Knives, Wilmington, DE) and placed in heated ACSF (37° C) for 40 min before transferring to room-temperature ACSF for at least 20 min before recording. ACSF contained (in mM): 126 NaCl, 3.5 KCl, 1.2 NaH_2PO_4 , 1.3 MgCl_2 , 2 CaCl_2 , 26 NaHCO_3 , 10 D-glucose. Sucrose ACSF contained (in mM): 210 sucrose, 2.5 KCl, 7 MgSO_4 , 1.5 NaH_2PO_4 , 1 CaCl_2 , 26 NaHCO_3 , and 10 D-glucose. The pH of ACSF and sucrose ACSF was 7.2–7.4 after saturation with 95% O_2 /5% CO_2 at room temperature, and the osmolarity was 310–320 mOsm.

All drugs, except for 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), were dissolved in distilled water and then added to the ACSF inflow line to achieve their final concentrations. Baclofen (10 μM ; racemic mixture; Sigma, St. Louis, MO) is a specific GABA_B receptor agonist. Tetrodotoxin (1 μM ; TTX; Alomone Labs, Jerusalem, Israel) blocked voltage-dependent sodium channels and revealed the miniature synaptic events. Bicuculline methochloride (10 μM ; Tocris, St. Louis, MO) blocked GABA_A receptor-mediated events, strychnine (1 μM ; Sigma) blocked glycine receptor-mediated events, and AP-5 (30 μM ; Sigma) blocked NMDA receptor-mediated events. CNQX (RBI, Natick, MA) was dissolved in dimethyl sulfoxide (20 mM; Fisher Scientific, Fair Lawn, NJ) and then added to the ACSF inflow line to achieve the final concentration (10 μM). CNQX blocked AMPA/KA receptor-mediated events.

Most drugs were applied using protocols and concentrations from previous studies of the tangential principal cells (Shao et al., 2003; 2004). The concentration of baclofen was selected based on a study of rat brain slices which found that 10 μM baclofen abolished 50–60% of the resting spike discharge of rostral MVN neurons after 60 seconds (Yamanaka et al. 2000). Indeed, along with 20 or 50 μM baclofen, 10 μM baclofen represents a standard dose to record electrophysiological responses in brain slices (Wang et al., 2007; Li et al., 2008). Thus, 10 μM baclofen was thought to produce major activation of GABA_B receptors in the tangential nucleus in brain slices. In all recordings, baclofen was added to the inflow line for three-to-four minutes before recording and continued during the three minutes of the recordings. The frequency of events recovered to control values in most recorded principal cells about 6–8 minutes after the baclofen was washed out of the recording chamber.

Electrophysiological techniques and data acquisition and analysis

The recording chamber, recording conditions, and recording pipets have been described (Shao et al., 2003; 2004). The recording pipets (2–5 $\text{M}\Omega$) were filled with either KCl or

cesium gluconate (Cs-gluconate) solution. KCl pipet solution contained (in mM): 130 KCl, 10 EGTA, 10 HEPES, 1.0 CaCl₂, and 2.0 Mg-ATP. The pH of the solution was adjusted to 7.2 with KOH (8.0 N) (Gamkrelidze et al., 1998). Cs-gluconate pipet solution contained (in mM): 135 Cs-gluconate, 1 EGTA, 10 HEPES, 0.1 CaCl₂, and 2 MgCl₂. The pH was adjusted to 7.2 with CsOH (Shao et al., 2003), and the osmolarity was adjusted to 270–290 mOsm. All the recording pipets contained 0.5% biocytin (Sigma) which was dissolved in the pipet solution weekly and kept frozen between experiments.

Electrophysiological signals were acquired and processed using Axopatch-200B amplifier or Multiclamp 700B (Molecular Devices, Foster City, CA) and Clampex 9.2 or 10.2 computer program (Molecular Devices) (for details, see Shao et al., 2012). Postsynaptic GABA_B receptor-mediated currents were recorded using KCl pipet solution, while the effect of presynaptic GABA_B receptors on spontaneous synaptic events in principal cells was recorded using Cs-gluconate pipet solution. Based on reversal potentials, miniature excitatory postsynaptic currents (mEPSCs) were recorded at a holding potential of -60 mV, while miniature inhibitory postsynaptic currents (mIPSCs) were recorded at +10 mV (Shao et al., 2003). Typically, spontaneous synaptic events were collected continuously for 3 minutes for each recorded neuron, first at +10 mV and then at -60 mV. No corrections were made for the voltage offset observed after withdrawing the pipet from the cell (± 3 mV) or liquid junction potential of the pipet solution (+3 mV). Synaptic currents were analyzed off-line using Minianalysis 6.0.3 computer program (Synaptosoft, Decatur, GA). The threshold for acceptable events was 3x root mean square of baseline noise, which was 2–4 pA at -60 mV and 4–7 pA at +10 mV. Thus, between 50 and 200 events were analyzed for each recorded neuron under each experimental condition at each holding potential. The frequency and kinetics of postsynaptic currents were analyzed, including peak amplitude and decay time (from 90 to 37% amplitude return to baseline). To determine whether the fluctuations in frequency and kinetics of excitatory and inhibitory spontaneous synaptic events produced an overall change in excitability of the principal cells after the lesions, synaptic charge transfer was calculated (Dani et al., 2005; Han and Stevens, 2009; Shao et al., 2012). Synaptic charge transfer was calculated by integrating all the synaptic events from baseline, but maintaining separate calculations for the excitatory and inhibitory events recorded from each principal cell (Gao et al., 2011). The percent decrease in miniature synaptic events after baclofen exposure was calculated for each neuron and then averaged to obtain the percent decrease for the experimental group.

Visualization of recorded and biocytin-injected principal cells

All the recorded neurons were filled passively with biocytin from the recording pipet during the recordings and then processed to confirm their identity as principal cells using a standard laboratory protocol (Shao et al., 2003). Biocytin-labeled neurons were visualized on a Zeiss LSM 710 confocal laser scanning microscope (Zeiss Instruments) equipped with 10x Plan Apochromat (NA, 0.45) and 20x PlanApo (NA, 0.8) objectives, as previously described (Shao et al., 2012).

Animal perfusion, fixation and tissue processing for immunolabeling

To extend the electrophysiological findings, GABA_BR2 immunolabeling was performed in controls, compensating, and uncompensated chickens three days after UVG. Under deep anesthesia, H7 control and operated chickens were perfused via the ascending aorta, first with a brief flush of 0.1 M phosphate buffered saline (PBS) for 1 minute, followed by 1% paraformaldehyde (PF) fixative in 0.1 M phosphate buffer for 20 minutes. After perfusion fixation, the chickens were decapitated and the brains removed and immersed in 1% PF for 12 hours in the refrigerator. On the following day, the brains were immersed in PBS just before sectioning. The portion of the brainstem containing the tangential nucleus was glued to the stage of a Vibratome (Oxford) and 50 µm transverse sections were cut in ice cold, 0.1 M PBS. Sections containing the tangential nucleus were identified under a dissecting microscope and processed immediately for immunolabeling.

Synaptotagmin 1 (Syt1) and synaptotagmin 2 (Syt2) immunolabeling

Synaptotagmin (Syt) is a family of synaptic vesicle membrane integral proteins with three isoforms, Syt1, Syt2, and Syt9, functioning as calcium sensors for fast synaptic vesicle release in neurons (Mittelstaedt et al., 2009). While individual neurons can express multiple Syt isoforms, they can express either Syt1, Syt2, or Syt9. Syt1 shows its highest expression in forebrain and midbrain terminals and lower expression in brainstem terminals (Geppert et al., 1991), whereas Syt2 predominates in spinal cord, brainstem, and cerebellar terminals (Geppert et al., 1991; Fox and Sanes, 2007; Mittelstaedt et al., 2009), and Syt9 is not expressed in brainstem terminals. Accordingly, here both Syt1 and Syt2 immunolabeling was performed in serial sections to reveal the full complement of terminals in the tangential nucleus.

Protocol for double immunolabeling of terminals with GABA_BR2 and Syt1 or Syt2

GABA_B receptors consist of two subunits, GABA_BR1 and GABA_BR2, which assemble as heteromers integrated into the neuronal plasma membranes to form functional metabotropic receptors (Jones et al., 1998; Kaupmann et al., 1998a; White et al., 1998; Kuner et al., 1999; Ng et al., 1999). Virtually all membrane-bound GABA_BR1 is associated with GABA_BR2. While GABA_BR1 exists as two isoforms, GABA_BR2 is present only as one. Accordingly, GABA_BR2 was selected for immunolabeling and combined with confocal imaging to obtain high resolution images of postsynaptic GABA_BR2 expression on MAP2-labeled principal cell bodies and presynaptic GABA_BR2 expression in Syt-labeled terminals in the tangential nucleus from control, compensating and uncompensated chickens three days after UVG.

Our immunolabeling procedure allowed for simultaneous observation of two immunolabels under the confocal microscope. Therefore, alternating brainstem sections containing the tangential nucleus were immunolabeled simultaneously with the primary antibodies, guinea pig anti-GABA_BR2 (IgG) (Millipore, AB5394) combined with either the primary antibody for mouse anti-microtubule associated protein 2 (MAP2) IgG (Millipore, MAB3418) to label neuron cell bodies and dendrites, or primary antibody for mouse anti-Syt1 IgG (Millipore, MAB5200) or primary antibody for mouse anti-Syt2 (ZNP1, ZIRC, Eugene, OR) to label synaptic terminals. The primary antibody for GABA_BR2 was revealed with a secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, A11073), goat anti-guinea

pig IgG, while anti-MAP2, anti-Syt1 and anti-Syt2 were revealed with secondary antibodies conjugated to goat anti-mouse IgG conjugated to Alexa Fluor 647 (Invitrogen, A21235). Sections double-labeled with GABA_BR2 and Syt1 or Syt2 were processed separately due to the incompatibility of Syt1 and Syt2 monoclonal antibodies for double Syt labeling.

Since fixation was performed using low concentration 1% PF fixative, it was unnecessary to permeabilize the tissue sections with Triton X-100. All steps in the tissue processing were performed in 0.1M PBS. After extensive rinsing in PBS, alternating sections were incubated in 10% normal goat serum (NGS) in PBS for 30 minutes, followed by incubation for 12-14 hours in a primary antibody cocktail including anti-GABA_BR2 (1:1000) combined with either anti-MAP2 (1:500), anti-Syt1 (1:400), or anti-Syt2 (1:400). On the next day, the sections were rinsed in PBS and incubated in 2% NGS in PBS for 10 minutes. Then, the sections were incubated for two hours in a cocktail of secondary antibodies, including goat anti guinea pig IgG conjugated to Alexa Fluor 488 and goat anti-mouse IgG conjugated to Alexa Fluor 647 (1:200). Finally, the sections were rinsed and mounted on glass slides, air dried, and coverslipped with anti-fading, water-soluble medium (Fluoromount G; Electron Microscopy Sciences). The sections were stored in the refrigerator and the images were visualized using confocal microscopy within two weeks.

Controls

Positive and negative controls were performed to confirm the specificity of immunolabeling and determine whether nonspecific binding of the primary and secondary antibodies used to reveal GABA_BR2, Syt1, Syt2, and MAP2 was apparent in tissue sections of the hatchling chicken under the same conditions used to capture images for analysis of immunolabeling (Fig. 1). To measure pixel brightness of the immunolabeling in controls, line scans were drawn along presumptive principal cell bodies, cerebellar Purkinje cell bodies, and/or Syt-labeled terminals in the cerebellum. The line scan was drawn in negative controls by boosting the pixel brightness of the image (Fig. 1).

As positive controls for GABA_BR2, Syt1 and Syt2 immunolabeling, transverse sections of the cerebellum were prepared. Strong GABA_BR2 immunolabeling was detected in subsets of cerebellar Purkinje cell bodies and their dendrites in the molecular layer, as described for mouse cerebellum (Chung et al., 2008) (Fig. 1A). In addition, Syt1-labeling was abundant in terminals in the molecular layer (Fig. 1B), while Syt2-labeling was abundant in terminals surrounding the Purkinje cell bodies (Fig. 1C), as found in the mouse cerebellum (Fox and Sanes, 2007).

Since blocking peptides for GABA_BR2 IgG were not available (Millipore, AB5394), two negative controls for GABA_BR2 immunolabeling were performed: (1) omitting the primary antibody and (2) cross-over labeling experiments in which the primary antibody was paired with an inappropriate secondary antibody which was used here to reveal other IgGs. First, the primary antibody for GABA_BR2 was omitted and sections of the tangential nucleus or cerebellum were incubated in 2% NGS followed by the appropriate secondary antibody for GABA_BR2 (Fig. 1D). No GABA_BR2 labeling was detected in the image (Fig. 1D₁). Second, cross-over control experiments were performed by incubating the tissue sections in primary

antibody for GABA_BR2 and then with secondary antibodies for MAP2, Syt1 or Syt2 (Fig. 1E). In all negative controls, no specific labeling for GABA_BR2 was detected.

Negative controls for the specificity of primary antibodies against MAP2 and Syt1 were performed in a previous study (Popratiloff et al., 2004), so here we investigated the specificity of primary antibodies against Syt2 by incubating tissue sections of the tangential nucleus in 2% NGS instead of primary antibodies for Syt2, followed by the appropriate secondary antibody, which produced no labeling (not shown). Finally, cross-over control experiments were performed by incubating primary antibodies for Syt1, Syt2, and MAP2 made in the mouse with secondary anti-guinea pig IgG used to detect GABA_BR2 immunolabeling. In all of these crossover control experiments, no specific labeling for MAP2, Syt1, or Syt2 was detected (not shown).

Confocal imaging

Confocal images (2048 × 2048 pixels; 135 × 135 μm) were collected on a Zeiss LSM 710 system (Carl Zeiss Microimaging GmbH), equipped with Axio Examiner Z1 upright microscope, Plan-Apochromat 63× oil DIC M27 objective (pixel resolution, 0.06 μm at 63× magnification; NA, 1.40), and Zen 2010 software. Alexa Fluor 488 was excited with the Argon 488 line of a multiline 25 mW argon laser, whereas Alexa Fluor 647 was excited with 5 mW HeNe emitting at 633 nm. The microscope was equipped with Prior x/y/z scanning stage, which permitted capturing stacks of images of the principal cell bodies and tangential nucleus neuropil. Alexa Fluor 488 was viewed on the red channel, while Alexa Fluor 647 was displayed on the green channel. Immunolabeling was adjusted within the image dynamic range using the gain control of the photomultiplier and consistency was maintained between images by saturating a minimum number of pixels at 0 and 255.

Confocal image analysis

Quantification of presynaptic GABA_BR2 immunolabeling in Syt1- and Syt2-labeled terminals and postsynaptic GABA_BR2 immunolabeling in MAP2-labeled principal cell bodies was performed using Image Pro Plus software with its segmentation protocol and count objects function (v. 7.0, Media Cybernetics, Bethesda, MD, USA). Although color changes occurred where overlap of GABA_BR2 immunolabeling was present in Syt1- and Syt2-labeled terminals, these changes were apparent only in high magnification confocal images (see Results). Images were selected for analysis from a confocal image stack based on the presence of at least one principal cell body with a visible nucleus. The number of images analyzed per stack depended on the number of images containing at least one principal cell body with a visible nucleus. The captured area in each image was confined completely within the boundaries of the tangential nucleus, and the images were obtained from all parts of the tangential nucleus across its anteroposterior, dorsoventral, and mediolateral dimensions.

Quantification of postsynaptic GABA_BR2 immunolabeling in MAP2-labeled principal cells—Independent thresholds for GABA_BR2 immunolabeling (red channel) and MAP2 immunolabeling (green channel) were set for each image analyzed based on the background intensity of the cell nucleus (Popratiloff et al., 2003; Shao et al., 2009) and were

equal to the mean plus $3 \times$ the standard deviation of the background intensity (Suzuki and Bekkers, 2010; Steen et al., 2007). The thresholds were determined from the nucleus of a single principal cell body present in the section. If multiple principal cell bodies were present, that cell with a nucleus cut through its largest plane in the optical section on the MAP2 channel was selected to set the threshold for the image. Next, by viewing separately the MAP2 channel, each identified principal cell body was outlined along its surface to limit the analysis to the cell body. Finally, the region of interest for each cell was transferred to the GABA_BR2 channel and the threshold for each channel was applied, so that the mean pixel brightness (0-255) was obtained for GABA_BR2 labeling in the MAP2-labeled principal cell body cytoplasm.

Surface expression of GABA_BR2 in MAP2-labeled principal cells—To evaluate GABA_BR2 expression on the surfaces of the principal cell bodies, a one-pixel thick line (0.06 μm at $63\times$ magnification; NA, 1.40) was drawn along the surface of the principal cell bodies identified by the presence of MAP2 labeling. The line was transferred to the GABA_BR2 channel where pixel brightness of GABA_BR2 labeling along the line was calculated and plotted. Also, lines were drawn at random in the cytoplasm and in the cell nucleus of each sampled principal cell body and transferred to the GABA_BR2 channel. The mean pixel brightness of the line scans from the surface and from the cytoplasm was normalized by subtracting the mean pixel brightness of the line scans from the cell nucleus (Shao et al., 2009).

Quantification of presynaptic GABA_BR2 labeling in Syt1- and Syt2-labeled terminals—In double-labeling experiments applying GABA_BR2 immunolabeling, using Syt1-labeling as the synaptic terminal marker, images (135 $\mu\text{m} \times 135 \mu\text{m}$) were taken of the tangential nucleus from control (n=35 images), lesion (n=20 images) and intact sides (n=29 images) of the compensating chickens three days after UVG, and the lesion (n=34 images) and intact sides (n=81 images) of uncompensated chickens three days after UVG. In parallel experiments, GABA_BR2 immunolabeling was performed using the Syt2-labeling as a synaptic terminal marker, and images (135 $\mu\text{m} \times 135 \mu\text{m}$) were taken of the tangential nucleus from control (n=55 images), lesion (n=35 images) and intact sides (n=53 images) of compensating chickens three days after UVG, and the lesion (n=32 images) and intact sides (n=46 images) of uncompensated chickens three days after UVG. Independent thresholds for GABA_BR2 immunolabeling (red channel) and Syt1 or Syt2 immunolabeling (green channel) were established based on background intensities of the nucleus and cytoplasm of the principal cells, respectively. Thresholds were equal to the mean plus $3 \times$ the standard deviation of the background level (Suzuki and Bekkers, 2010; Steen et al., 2007). The cell cytoplasm rather than the cell nucleus was used to determine the background level for the Syt1 and Syt2 channels because the principal cell body cytoplasm contained weak, uniform, but non-punctate labeling for Syt1 and Syt2, which was more intense than that in the nucleus but dramatically less intense than that found in the terminals (Popratiloff and Peusner, 2011). To confine the analysis to immunolabeling of the terminal fraction, the Syt threshold excluded labeling in the principal cell body cytoplasm. The threshold for the green channel was applied to determine Syt1- or Syt2- terminal area. Finally, the thresholds for both channels were combined to determine the mean pixel brightness of GABA_BR2

immunolabeling overlapping with Syt1- or Syt2-labeled presynaptic terminal profiles. According to this procedure, immunolabeled fibers and cell bodies were excluded from the analysis. Since Syt immunolabeling revealed only the synaptic vesicles in the terminals rather than the entire terminal, in principle, the “terminal area in the tangential nucleus” may be a lower estimate of the terminal area.

In preliminary experiments, perisomatic terminals expressing GABA_BR2 were counted and compared to the GABA_BR2-labeled terminals in the total tangential nucleus, which included terminals in the neuropil and those surrounding the principal cell bodies. No significant differences were detected in these two measures, indicating no specific subcellular termination pattern for GABA_B receptors. Therefore, the data presented here represents the total number of terminals in the tangential nucleus, including neuropil and perisomatic terminals.

Statistical analysis

All data were presented as mean±S.E.M. Wilcoxon matched pairs test was used to determine if the effect of baclofen was significant in changing the frequency of miniature synaptic events in principal cells within the same experimental group, whereas the Student t-test was used to determine if the effect of baclofen was significant in changing the frequency in principal cells from different experimental groups. The immunolabeling data were compared from different experimental groups using a one way ANOVA with the Bonferroni multiple comparison post-test. Statistical significance was set at $P<0.05$ for all experiments.

Results

Behavioral deficits in hatchling chickens after UVG

The survival rate after UVG was 95%. Briefly, within three to four hours after surgery, the operated hatchlings recovered from the anesthesia, but could not stand with asymmetric muscle tone in the lower extremities bilaterally. One day after surgery, the operated chickens could not stand, eat or drink, showing flexion of the ipsilateral leg and extension of the contralateral leg and extensive roll head tilt from 90° to 180° toward the side of the lesion. Roll head tilt is defined as the lateral deviation of the head and neck in the transverse plane. At one day after UVG, compensating and uncompensated chickens were indistinguishable, with the very first signs of recovery occurring between 1.5-2.5 days after UVG. At three days after UVG, 53% (34/64) of the operated chickens could stand and peck at food independently for extended periods, the “compensating” chickens, while the remainder, the “uncompensated chickens” failed to show any signs of recovery. However, the compensating chickens retained a 30° roll head tilt toward the lesion side. The uncompensated chickens showed no signs of recovery from the lesion even five days after UVG (not shown). All the operated hatchlings lost weight compared to the normal, age-matched controls due to their difficulty in feeding and drinking. However, by 56 days after UVG, control and operated chickens are indistinguishable in weight (Aldrich and Peusner 2002). The observed behavioral deficits after UVG, the time course for recovery from the static symptoms in compensating chickens, and the percentages of compensating and

uncompensated chickens were similar here as found previously (Aldrich and Peusner, 2002; Shao et al., 2009; 2012).

Qualitative tests for VOR function included examining the operated hatchlings for spontaneous nystagmus, tonic eye deviation, whether the eyes were open or closed while the animal is awake, and the ability to peck at food. On visual inspection, spontaneous nystagmus and tonic eye deviation were not observed immediately after UVG. However, by 12 hours after surgery, some animals kept one eye open and the other eye closed, while others have both eyes open while awake. By one day after UVG, all operated hatchlings kept both eyes open while awake. Unlike other species, oculomotor abnormalities were especially difficult to detect in the hatchlings because they normally exhibit 1–5° amplitude saccades every one to three seconds (Wallman et al., 1982). Nonetheless, an impaired VOR was likely in the operated chickens due to their difficulty in pecking at food.

Principal cell morphology, passive membrane properties and spontaneous spike firing

Whole-cell voltage-clamp recordings were obtained from 114 morphologically-identified principal cells, including those from controls (n=22), lesion (n=15) and intact side (n=14) of operated chickens one day after UVG, lesion (n=18) and intact side (n=16) of compensating chickens three days after UVG, and the lesion (n=13) and intact side (n=16) of uncompensated chickens three days after UVG. The morphology, resting membrane potential, input resistance and spontaneous spike firing under cell-attached configuration of the principal cells were similar in the present study to those described previously (Shao et al., 2009, 2012). Briefly, the principal cells have large, oval cell bodies with axons originating usually from the medial surface of the cell body and three primary dendrites which most often radiate in the dorsal and lateral directions (not shown). Thus, the major reason for labeling the principal cells was to validate their identity and insure that the data were obtained and averaged from the same population of vestibular nuclei neurons.

In controls, the average input resistance of the principal cells was $147 \pm 14.8 \text{ M}\Omega$ (n=22), with no difference between the spontaneous spike firing and silent principal cells, which could not fire spikes in cell-attached mode. One day after UVG, the input resistance did not change significantly in principal cells on the lesion or intact side, but by three days, the input resistance was significantly higher for those neurons on the lesion side ($257.1 \pm 19.8 \text{ M}\Omega$, n=31) compared to the intact side ($121.5 \pm 9.5 \text{ M}\Omega$, n=32) in all operated animals, which may be due to the dendritic shrinkage previously described (Shao et al., 2009). In controls, 45% (n=10/22) of the principal cells generated spontaneous spike firing in cell-attached recordings, and showed a mean spike firing rate of 21.3 ± 4 spikes/sec (n=10), with a range of 9.8–36.1 spikes/sec. After UVG, the percentage of spike-firing principal cells decreased significantly on the intact side. About 14% of principal cells fired spikes on the intact side one day and three day after UVG in compensating chickens (n=2/14 at one day; n=2/16 at three days), while none of the principal cells fired spikes on the intact side of the uncompensated chickens by three days after UVG (n=0/16).

Expression of postsynaptic GABA_B receptors

To determine whether postsynaptic GABA_B receptors were active in principal cells from H7 controls, whole-cell voltage-clamp recordings were performed using KCl pipet solution to characterize the GABA_B receptor-mediated postsynaptic currents. Also, immunolabeling combined with confocal imaging experiments were performed to quantify the GABA_B expression in the cytoplasm and on the surface membranes of the principal cell bodies. Baclofen was added to the ACSF solution for 90 seconds at a holding potential of -65 mV, along with 1 μ M TTX, 10 μ M bicuculline, 1 μ M strychnine, 30 μ M AP-5, and 10 μ M CNQX. On exposure to baclofen (10 μ M), no outward postsynaptic GABA_B receptor-mediated currents were detected in the principal cells (n=7) (Fig. 2A), even at a higher concentration (30 μ M) (n=2). In contrast, using the same recording conditions and 10 μ M baclofen, a small, stable outward current of 10-40 pA is induced in principal cells at E16 (Shao et al., 2003). Accordingly, a developmental down-regulation of the GABA_B receptor-mediated postsynaptic current occurred in principal cells between E16 and H7.

To confirm the absence of GABA_B receptor expression in principal cells—

GABA_BR2 immunolabeling of the principal cell bodies was performed in controls and compensating chickens three days after UVG. In controls, cytoplasmic expression was detected in all MAP2-positive, principal cell bodies (Fig. 2B-D). GABA_BR2 labeling appeared to be uniform throughout the principal cell body, but the labeling weakened a few microns from the cell surface. In controls, the mean pixel brightness for GABA_BR2 cytoplasmic immunolabeling was 46.2 ± 1.7 (n=109 cell body profiles) (Fig. 2K). In compensating chickens, the mean pixel brightness of GABA_BR2 immunolabeling was 51.2 ± 1.7 in principal cell bodies on the lesion side (n=46) and 57.1 ± 3.0 in principal cell bodies on the intact side (n=36), with a significant increase on the intact side compared to controls (Fig. 2E-J and K) ($P < 0.01$). In contrast to somatic GABA_B immunolabeling, GABA_BR2 labeling in MAP2-labeled dendrites in the tangential nucleus was not uniform. While the dendrites were labeled uniformly with MAP2, they contained regions which were weakly labeled or failed to label with GABA_BR2 (Fig. 2E-G).

The cytoplasmic expression of GABA_BR2 in the principal cell bodies from controls and compensating chickens indicated the presence of synthetic and translocating pools, but did not demonstrate functionally-active subunits, which could be supported by finding GABA_BR2 surface expression on the plasma membranes of the principal cell bodies. To measure surface expression, line scans of selected lengths were drawn in the principal cell body cytoplasm and along the surface membranes. The mean pixel brightness for GABA_BR2 immunolabeling on line scans drawn in the cytoplasm was lower than that detected within the cytoplasm ($P < 0.01$) because different measures were used to obtain the data (See Methods). However, the two approaches revealed similar patterns of increased pixel brightness for GABA_BR2 immunolabeling in principal cells on the intact side of compensating chickens compared to controls (Figs. 2K, 2L; Table 1). We found that the mean pixel brightness of GABA_BR2 immunolabeling on the surface of the principal cell bodies was significantly weaker than that measured in the principal cell cytoplasm in all experimental groups ($P < 0.01$), indicating there was less GABA_BR2 localized on the cell surface relative to the cytoplasm. In addition, the mean pixel brightness of the line scans

drawn on the surfaces of the principal cell bodies did not change significantly between controls and compensating chickens three days after UVG (Fig. 2L, Table 1).

To measure the efficiency of GABA_BR2 surface expression relative to the cytoplasmic synthetic pool, ratios were calculated for the line scans drawn on the cell surface and in the principal cell body cytoplasm. These ratios were similar in controls and compensating chickens (Table 1). Thus, despite increased GABA_BR2 immunolabeling in the cytoplasm of the principal cell bodies on the intact side of compensating chickens three days after UVG, no change was detected in GABA_BR2 surface expression. Taken together, the electrophysiology and immunolabeling results support that there is little, if any, functional postsynaptic GABA_B activity in the principal cells in controls or compensating chickens.

Expression of presynaptic GABA_B receptors

To determine whether presynaptic GABA_B receptors were present in terminals contacting the principal cells, and, if so, which terminal types have GABA_B receptors, miniature spontaneous synaptic events were recorded from principal cells in controls, the lesion and intact sides of hatchlings one day after UVG, and the lesion and intact sides of compensating and uncompensated chickens three days after UVG. Frequency, amplitude, decay time, and synaptic charge transfer of the excitatory and inhibitory synaptic events were measured. On exposure to baclofen, changes in frequency and synaptic charge transfer were calculated and compared in principal cell in different experimental groups. Since baclofen decreased the frequencies of miniature synaptic events without modifying their kinetics (see below), changes in frequency and synaptic charge transfer ran in parallel. Accordingly, the data are presented here only for frequency of events. In addition, since the miniature excitatory and inhibitory events on baclofen exposure did not change significantly one day after UVG compared to controls, the data were tabulated without including them in the text (Table 2).

Using Cs-gluconate pipet solution in voltage-clamp recordings—mEPSC and GABAergic mIPSC frequencies in the principal cells always decreased significantly on exposure to baclofen, regardless of the experimental group (Wilcoxon matched pairs test, $P < 0.05$). These findings support the presence of presynaptic GABA_B receptors and their active involvement in synaptic transmission at glutamatergic and GABAergic terminals contacting the principal cells in brain slices before and after UVG. Specifically, on exposure to 10 μ M baclofen, mEPSC frequency decreased 26% in principal cells in controls, from 1.2 ± 0.4 Hz to 0.8 ± 0.2 Hz ($n=12$) (Fig. 3A₁, A₂, C and D; Table 2). Three days after UVG, the percentage decrease in mEPSC frequency on baclofen exposure was greatest in principal cells on the intact side of *uncompensated* chickens (48%) compared to controls and the lesion sides of compensating and uncompensated chickens (Fig. 3D) ($P < 0.05$). Indeed, baclofen decreased mEPSC frequency in principal cells on the intact side of uncompensated chickens three days after UVG from 1.5 ± 0.4 Hz to 0.8 ± 0.2 Hz ($n=10$) (Fig. 3B₁, B₂, C and D; Table 2). It is interesting that in the present and previous study, after UVG the frequency of excitatory events increased most prominently in principal cells on the lesion side of uncompensated chickens (Fig. 3C; Table 2) (Shao et al., 2012).

GABAergic and glycinergic mIPSCs were distinguished by adding specific neurotransmitter antagonists (see Methods). On exposure to baclofen, GABAergic mIPSC frequency in principal cells in controls decreased 27% from 4.2 ± 2.5 Hz to 2.9 ± 1.7 Hz ($n=7$) (Fig. 4C, D; Table 2). The percentage decrease in GABAergic mIPSC frequency after baclofen exposure was greatest in principal cells on the intact side of *compensating chickens* three days after UVG (53%) compared to principal cells on the lesion side (Fig. 4D) ($P<0.05$). Indeed, baclofen decreased GABAergic mIPSC frequency from 1 ± 0.5 Hz to 0.4 ± 0.2 Hz ($n=7$) on the intact side, but only 28% from 8.2 ± 3.3 Hz to 6.8 ± 2.9 Hz on the lesion side ($n=11$) (Fig. 4; Table 2).

Unlike mEPSC and GABAergic mIPSC frequency, exposure to baclofen decreased glycinergic mIPSC frequency significantly in principal cells on the intact side one day and three days after UVG in compensating chickens (Wilcoxon matched paired test; $P<0.05$). Indeed, on exposure to 10 μ M baclofen, glycinergic mIPSC frequency in principal cells in controls decreased 8% from 0.79 ± 0.19 Hz to 0.68 ± 0.16 Hz ($n=7$) (Fig. 5; Table 2), with similar small decreases in principal cells in other experimental groups (Fig. 5C) (Student t -test; $P>0.05$). Moreover, the percentage decrease in glycinergic mIPSC frequency on baclofen exposure did not change significantly in principal cells when comparing different experimental groups. Apparently, glycinergic terminals are not involved in GABA_B-mediated recovery of function after UVG. Finally, baclofen did not change the kinetics of mEPSCs, GABAergic or glycinergic mIPSCs in principal cells from any experimental group (Table 2). Since kinetics are determined primarily by postsynaptic factors, this finding confirmed that GABA_B receptors acted mainly at presynaptic terminals contacting the principal cells.

GABA_BR2 and Syt double-labeled terminals in the tangential nucleus—To confirm and extend the electrophysiological findings, double-labeling for GABA_BR2 and Syt1- or Syt2- was performed on the tangential nucleus from controls, compensating and uncompensated chickens three days after UVG. The expression of presynaptic GABA_B receptors was measured by analyzing pixel brightness of GABA_BR2 immunolabeling (gray scale 0-255) in Syt1- and Syt2-labeled terminals and the percentage of Syt1- and Syt2-labeled terminal area overlapping with GABA_BR2 immunolabeling. Syt labeling was used here to estimate the number of synaptic terminals in the tangential nucleus. In controls and operated chickens, GABA_BR2 immunolabeling overlapped with only a fraction of Syt1- and Syt2-labeled terminals in the tangential nucleus (Figs. 6A-Q and T; 7A-Q and T; Table 3). In fact, the double-labeled terminals appeared to have GABA_BR2 and Syt only partially overlapping with GABA_BR2 incorporated into the surface membranes and Syt localized to the synaptic vesicle fraction in the terminals.

Syt-labeled terminals in the tangential nucleus of controls—Since Syt1 and Syt2 represent functionally-distinct isoforms of Syt whose expression patterns are complementary in the brainstem and collectively label all brainstem terminals (e.g., Mittelstadt et al., 2009), Syt1- and Syt2-immunolabeling was performed on serial tissue sections throughout the tangential nucleus to label all of the synaptic terminals. In controls, Syt1- and Syt2-labeled terminals were present in the neuropil and on the principal cell bodies (Figs. 6A-Q; 7A-Q).

The mean Syt-labeled terminal area and percentage of total sampled area in the tangential nucleus was similar for Syt1- and Syt2-labeled terminal areas in controls (Figs. 6R, 7R; Table 3).

Syt-labeled terminals in the tangential nucleus after UVG—The number of terminals contacting a neuron is one factor which contributes to setting the frequency of spontaneous synaptic events (Li and Prince, 2002; Caillard, 2011). To address whether increased GABAergic mIPSC frequency recorded in principal cells on the lesion side of compensating chickens and increased mEPSC frequency recorded in principal cells on the lesion side of uncompensated chickens three days after UVG were due to increased terminal number, the amount of Syt1- and Syt2-positive terminal area in the tangential nucleus was measured. In compensating chickens, there was no significant difference in the amount of *Syt1- or Syt2-labeled total terminal area* on the lesion or intact sides relative to controls, suggesting that *there was* neither a major gain nor loss of terminals in the tangential nucleus of this experimental group (Figs. 6R, 7R; Table 3). However, in the uncompensated chickens, a dramatic loss of both Syt1- and Syt2-labeled terminal area was detected on both the lesion and intact sides of the tangential nucleus (Figs. 6R, 7R; Table 3) ($P < 0.01$). Altogether, these results showed that the increased frequency of miniature events on the lesion side three days after UVG could not be explained by an increased number of synaptic terminals and must result from other factors.

Distribution of GABA_BR2 in Syt1- and Syt2-labeled terminals before and after UVG—In controls, the mean pixel brightness of GABA_BR2 in Syt1-labeled terminals in the total tangential nucleus was significantly higher than that found in Syt2-labeled terminals (Figs. 6S and 7S; Table 3). However, the percentage of Syt1-labeled terminal area overlapping with GABA_BR2 was significantly lower than that found for Syt2-labeled terminals (Figs. 6T and 7T; Table 3). Thus, in controls, GABA_BR2 receptors are more concentrated in Syt1-labeled terminals than in Syt-2 labeled terminals, but more Syt2-terminals express GABA_BR2 than Syt-1 terminals in the tangential nucleus.

In compensating chickens three days after UVG, the mean pixel brightness of GABA_BR2 in Syt1-labeled terminals and the percentage of Syt1-labeled terminal area overlapping with GABA_BR2 were similar to controls ($P > 0.05$) (Fig. 6S, 6T; Table 3), indicating that there was no change in concentration of presynaptic GABA_BR2 or percentage of terminals expressing GABA_BR2 in Syt1-labeled terminals after UVG. However, the mean pixel brightness of GABA_BR2 in Syt2-labeled terminals and the percent of Syt2-labeled terminal area overlapping with GABA_BR2 increased significantly on the intact side compared to the lesion side of compensated chickens after UVG ($P < 0.05$) (Fig. 7S, 7T; Table 3). Taken together, these results indicated significant increase in presynaptic GABA_BR2 concentration and percentage of Syt2-labeled terminals which express GABA_BR2 on the intact side of compensating chickens, and suggested that the greatest decrease in GABAergic mIPSC frequency found in principal cells on the intact side of compensating chickens after baclofen exposure was due to increased GABA_B autoreceptor expression in a large fraction of Syt2-labeled terminals. Thus, a fraction of Syt2-labeled terminals which are GABAergic on the

intact side undergo increased GABA_B receptor expression by three days after UVG in the compensating chickens.

In uncompensated chickens three days after UVG, the mean pixel brightness of GABA_BR2 immunolabeling in Syt1-labeled terminals increased significantly on both the lesion and intact sides compared to controls and the lesion side and intact sides of compensating chickens (Fig. 6S, Table 3) ($P<0.01$). The percent of Syt1-labeled terminal area overlapping with GABA_BR2 increased significantly on the intact side compared to the lesion side of uncompensated chickens and controls ($P<0.05$) (Fig. 6T; Table 3). In uncompensated chickens, the mean pixel brightness of GABA_BR2 immunolabeling in Syt2-labeled total terminal area increased significantly on both the lesion and intact sides compared to controls (Fig. 7S) ($P<0.01$). However, the percent of Syt2-labeled terminal area overlapping with GABA_BR2 were similar to compensating chickens and controls (Fig. 7T; Table 3) ($P>0.05$). Thus, although the concentration of presynaptic GABA_BR2 increased in Syt1- and Syt2-labeled terminals on the lesion and intact sides of uncompensated chickens, only Syt1-labeled terminals on the intact side showed a concurrent increase in percentage of overlap with GABA_BR2-labeled terminals. Since the greatest decrease in mEPSC frequency after baclofen exposure was recorded on the intact side of uncompensated chickens, our results from the immunolabeling experiments indicated that both increased concentration of presynaptic GABA_B receptors and increased percentage of terminals expressing GABA_B receptor were necessary to produce the significant changes in GABA_B receptor function. Consequently, we propose that the increased GABA_B receptor function in glutamatergic terminals on the intact side of uncompensated chickens three days after UVG was due to increased GABA_B heteroreceptor expression in Syt1-labeled terminals.

Discussion

The effect of baclofen on spontaneous synaptic events in principal cells correlated well with the GABA_BR2 immunolabeling to provide strong evidence for an important role of presynaptic GABA_B receptor-mediated inhibition during early stages of vestibular compensation. The immunolabeling revealed widely-distributed GABA_B receptor expression in Syt1- and Syt2-labeled terminals in the tangential nucleus. In controls, presynaptic GABA_B receptors inhibited mEPSC and GABAergic mIPSC frequencies to a similar degree, but decreased glycinergic mIPSC frequency to a lesser extent. On baclofen exposure, compensating chickens three days after UVG showed the greatest percentage decrease in *GABAergic mIPSC frequency* in principal cells on the intact side, concurrent with increased GABA_BR2 pixel brightness and percentage overlap in Syt2-labeled terminals. In contrast, on baclofen exposure, uncompensated chickens showed the greatest percentage decrease in *mEPSC frequency* in principal cells on the intact side, concurrent with increased GABA_BR2 pixel brightness and percentage overlap in Syt1-labeled terminals. Finally, no postsynaptic GABA_B receptor-mediated currents were detected in the principal cells, consistent low GABA_BR2 surface expression. Altogether, these results revealed an exclusive presynaptic role for GABA_B autoreceptors in principal cells of the chicken tangential nucleus, with preferential up-regulation on the intact side of compensating chickens three days after UVG.

Absence of postsynaptic GABA_B activity

It was surprising that postsynaptic GABA_B receptor-mediated currents were not recorded in principal cells in H7 controls, since these currents are present at E16 (Shao et al., 2003) and postsynaptic baclofen binding sites have been identified in MVN neurons of monkey and rat (Holstein et al., 1992). The absence of postsynaptic GABA_B receptor-mediated currents in principal cells may be due to the failure of GABA_BR2 subunits to form membrane-bound, functional receptors at H7. Many factors could contribute to the lack of surface expression of functional GABA_B receptors, including the failure to localize on the cell surface, failure to form GABA_BR1 and GABA_BR2 heterodimers necessary for binding agonists (Calver et al., 2000; Margeta-Mitrovic et al., 2000; Galvez et al., 2001), and failure to associate with GIRK/Kir3-type potassium channels which generate GABA_B receptor-mediated currents (Kaupmann et al., 1998b; for review, see Bettler and Tiao, 2006). The immunolabeling studies showed a relative lack of GABA_BR2 surface expression in MAP2-labeled principal cell bodies from controls and compensating chickens, which is consistent with the absence of receptors localized on the cell surface. It is possible that the principal cells undergo a developmental switch in targeting GABA_B receptors from somatic to dendritic surfaces. This may explain the patchy appearance of GABA_BR2 labeling in some MAP2-labeled dendrites in the tangential nucleus (see Fig. 2E-G). This switch could also explain the large volume of GABA_BR2 immunolabeling detected in the cytoplasm of the hatchling principal cell bodies, and open up a new venue to explore the redistribution of GABA_B receptors in different cellular compartments during development and after peripheral vestibular lesions. However, if GABA_B receptors were translocated to the dendrites, postsynaptic GABA_B receptor-mediated currents would be recorded from dendrites within 300 μm of the cell body (Cossart et al., 2000). Currently, there is evidence for the formation and retention of homodimers of GABA_BR1 and GABA_BR2 in cultured cells (Fritschy et al., 2004) and GABA_BR2 expression in the absence of GABA_BR1 in the rat (Villemure et al., 2005), suggesting that GABA_BR1 and GABA_BR2 proteins may not always form functional, heteromeric receptors in vitro and in vivo. Thus, the presence of GABA_BR2 immunolabeling in the cell body does not confer functional postsynaptic GABA_B receptors on the cell surface. It remains to be seen what role postsynaptic GABA_BR2 play in the principal cells, if not functioning as GABA-sensitive, postsynaptic receptors. Finally, although GABA_B expression increased in the principal cell cytoplasm on the intact side of compensating chickens, surface expression remained low as in controls, indicating a minimal effect of postsynaptic GABA_B receptors in principal cells during vestibular compensation.

Increased frequency of spontaneous synaptic events was not due to increased number of terminals

After UVG, increased GABAergic mIPSC frequency was recorded on the lesion side of compensating chickens, and increased mEPSC frequency was recorded on the lesion side of uncompensated chickens in the present and previous study (Shao et al., 2012). Our results failed to show any increase in Syt1- and Syt2-labeled terminals in the tangential nucleus after UVG. In fact, dramatic and widespread *losses* were detected in Syt1- and Syt2-terminal area on the lesion and intact side of uncompensated chickens. Besides terminal number,

other factors can influence the frequency of miniature events, including the probability of presynaptic neurotransmitter release, number of postsynaptic receptors, and number of synaptic vesicles in the terminals.

Syt1 and Syt2 are proteins localized to synaptic vesicles, so reduced Syt immunolabeling could indicate loss of synaptic vesicles related to presynaptic terminal degeneration. Indeed, some loss of Syt immunolabeling was expected due to the degeneration of the primary vestibular fibers and their terminals on the lesion side after UVG (Aldrich and Peusner, 2002). However, loss of Syt1- and Syt2-immunolabeling in the uncompensated chickens was bilateral and equal in amounts on the lesion and intact side, suggesting that other factors besides vestibular deafferentation were involved. It was notable that Syt1- and Syt2-labeling was abundant in the brainstem regions neighboring the tangential nucleus (not shown), and no loss in Syt terminals was measured in the tangential nucleus of compensating chickens (Table 3). While the number of principal cells in the tangential nucleus are constant on the lesion and intact side of compensating chickens up to eight weeks after UVG (Aldrich and Peusner, 2002), it is not known whether the principal cells themselves degenerate in the uncompensated chickens.

Importance of miniature synaptic events

Here, the presynaptic action of baclofen was investigated by recording miniature synaptic events using Cs-gluconate pipet solution, which blocks GABA_B receptor-mediated postsynaptic potassium currents (Thompson et al., 2006). Miniature synaptic events were recorded so that GABA_B receptor activity could be investigated selectively on presynaptic terminals contacting the principal cells, and the global effects of baclofen on the neural circuitry outside the tangential nucleus were excluded. In other systems, miniature synaptic events are important because they modulate spike firing (Carter and Regehr, 2002), modify dendritic spine structure (McKinney et al., 1999; for review see Segal 2010), or regulate dendritic protein synthesis (Sutton et al., 2007). Miniature synaptic events can be calcium-independent, and may or may not be generated from the same synaptic vesicle pool which responds to action potentials (Sara et al., 2005; Neher and Sakaba, 2008). Thus, the effects of baclofen in the present study were due to calcium-independent, presynaptic mechanisms that involve G-protein interactions with protein kinase A (Couve et al., 2002), protein kinase C (Tremblay et al., 1995) and/or direct effects on the exocytotic mechanism (Capogna et al., 1996). Could the GABA_B effects reported here on miniature events be less important than the effects on action potential-driven, neurotransmitter release? It is likely that glutamate and GABA quantal release produces a considerable impact on network excitability, since spontaneous release of both neurotransmitters is mediated primarily by miniature synaptic events in the tangential principal cells of the hatchling chicken (see Fig. 4, Shao et al., 2012).

GABA_B receptor function

GABA_B receptors are one of a large array of presynaptic terminal receptors regulating neurotransmitter release. Presynaptic terminal receptors provide a highly sensitive mechanism to dynamically regulate synaptic transmission at synaptic sites and their associated networks. For example, baclofen decreases both GABA and glutamate release in

layer II and layer V neurons of the entorhinal cortex in normal rat brain slices. Decreased action of GABA_B heteroreceptors is thought to be instrumental in inducing epilepsy (Bailey et al., 2004; Thompson et al., 2006). In the cerebellar molecular layer, presynaptic GABA_B autoreceptors decrease GABA release from stellate cells onto Purkinje cells nearly 60% in two-week-old mice, but less than 20% in four-week-old mouse, suggesting that GABA_B autoreceptors are down-regulated during development (Astori et al., 2009).

Behavioral studies reveal that GABA_B agonists can accelerate recovery from the static and dynamic symptoms during early and later stages of vestibular compensation (Magnusson et al., 1998; 2000; 2002; Heskin-Sweezie et al., 2010). Studies on the cellular mechanisms underlying recovery from the static symptoms show that GABA_B receptor action is down-regulated in MVN neurons on the lesion side in rat brain slices four (Yamanaka et al., 2000) and 24 hours after UL (Johnston et al., 2001), which is thought to be responsible for their recovery of spike discharge. To explain increased spike discharge, GABA_B receptor down-regulation must occur primarily at presynaptic glutamatergic terminals and/or postsynaptic MVN neurons. In the tangential nucleus, presynaptic GABA_B receptors were also down-regulated on the lesion side, but at GABAergic terminals. Clearly, GABA_B receptor action differs in rat MVN and chick tangential nucleus during vestibular compensation, which could be due to the different types of lesions, the time after the lesion the experiments were performed, the species studied, and/or the functional class of vestibular nuclei neurons selected for study.

One day after UVG, the percentage decrease in frequency of glutamatergic and GABAergic events in principal cells on the lesion and intact side was similar on baclofen exposure, perhaps because incipient compensating and uncompensated chickens could not be distinguished and were investigated as one group. Thus, differences in their electrophysiological properties might cancel out by averaging the responses, as supported by the large SEM for the data. It would be intriguing to determine whether eye movements recorded from chickens one day after UVG would reveal different responses for potentially compensating and uncompensated chickens. The effect of baclofen on experimental animals which fail to compensate on schedule has not been studied. The relationship of the present data to behavioral recovery may be illuminated by injecting baclofen into the operated chickens one day after UVG to observe whether by three days changes occur in the number of compensating chickens, frequency of excitatory or inhibitory synaptic events, or synaptic charge transfer.

In the compensating chickens three days after UVG, GABAergic mIPSC frequency increased in principal cells on the lesion side compared to the intact side. Also, increased GABA_B expression decreased GABAergic mIPSC frequency to a greater extent in principal cell on the intact side by acting at GABAergic, Syt2-labeled terminals. GABA_B increased activity on the intact side would augment the differences in inhibitory frequency bilaterally (Bergquist et al., 2008). Based on the faster recovery which occurs in whole animals infused with baclofen after lesions, increased disparity in GABAergic release bilaterally must be beneficial for recovery from the static symptoms, although this conclusion is disputed (for review, see Dieringer, 2003). Further study is necessary to identify the neuronal source or sources of the inhibitory, Syt2-labeled terminals which undergo increased GABA_B receptor

expression after UVG. At present, Purkinje cells in the cerebellar flocculus represent a possible source of inhibitory terminals contacting the principal cells (Cox and Peusner, 1990), and also a potential source of Syt2-labeled terminals (Geppert et al., 1991; Fox and Sanes, 2007; Mittelstaedt et al. 2009).

In the uncompensated chickens three days after UVG, mEPSC frequency increased considerably in the principal cells on the lesion side compared to the intact side. In contrast to compensating chickens, presynaptic GABA_B receptors produced the greatest decrease in mEPSC frequency in principal cells on the intact side, which would augment the differences in excitatory frequencies bilaterally. How is increased glutamatergic synaptic transmission on the lesion side related to the failure to compensate? Clearly, increased excitatory input to the principal cells must occur to some extent to replace the lost excitatory input following degeneration of the primary vestibular terminals after UVG. In fact, mEPSC frequency is balanced bilaterally in compensating chickens, indicating increased excitatory inputs on the lesion side to offset the loss of the primary vestibular terminals. However, increased excitatory activity in uncompensated chickens is excessive on the lesion side and out of balance bilaterally. Although baclofen may facilitate recovery in the compensating subjects, it seems unlikely that baclofen could rescue the principal cells in uncompensated chickens. Indeed, the principal cells on the lesion side may be overexcited by inputs from the remaining vestibular circuitry whose firing patterns are modified by the abnormal behavior induced by the lesion. Finally, increased GABA_BR2 heteroreceptor expression was found in Syt1-labeled terminals on the intact side of the uncompensated chickens. The most likely source of glutamatergic, Syt1-labeled terminals is from neurons in higher brain centers (Geppert et al., 1991; Fox and Sanes, 2007; Mittelstaedt et al., 2009), including primary vestibular cortex. In fact, direct pathways from primary vestibular cortex to the vestibular nuclei have been found in primates (Kuypers and Lawrence, 1967; Akbarian et al., 1993; Guldin et al., 1993; Faugier-Grimaud and Ventre, 1989).

Does the Bechterew phenomenon explain these results in brain slice experiments?

The Bechterew phenomenon (1883) occurs when a second peripheral vestibular lesion is performed after some time has elapsed for recovery from the first lesion on the opposite side (Vibert et al., 1999; Beranek and Idoux, 2012). A delay of at least three days between the first and second lesion is usually required to activate the phenomenon. Immediately after the second lesion in the guinea pig, a higher percentage of MVN neurons are silent on the newly-deafferented side and neurons which fire spikes show decreased spike firing rates (Vibert et al., 1999). Theoretically, the second vestibular deafferentation would occur in the present study during brain slice preparation in a chicken that underwent UVG three days earlier. In fact, a higher percentage of silent principal cells are found on the newly-deafferented side and these neurons show decreased GABAergic mIPSC frequency and greater GABA_B autoreceptor inhibition in compensating chickens. Down-regulation of inhibitory events by GABA_B would assist in restoring excitability in the newly-deafferented principal cells. While the major electrophysiological findings could be explained by invoking the Bechterew phenomenon, it seems unlikely that the changes described here on the newly-deafferented side could occur within the time span of one-to-three hours between preparing the brain slices and recording from them. Moreover, the immunolabeling data,

which confirmed the electrophysiological data, were obtained from operated chickens which were fixed by perfusion without cutting the peripheral vestibular receptors on the intact side. Thus, the Bechterew phenomenon cannot explain the immunolabeling results.

Conclusions

Previous studies of GABA_B receptor expression failed to find differential changes in presynaptic and postsynaptic GABA_B expression after peripheral vestibular lesions (Eleore et al., 2005). However, by combining two highly-sensitive approaches, patch-clamp recording and immunolabeling visualized by confocal imaging, here we could readily distinguish between presynaptic and postsynaptic effects of GABA_B receptors in a morphologically-defined population of vestibular reflex projection neurons. Our results indicated a presynaptic role for GABA_B receptor-mediated inhibition of excitatory and inhibitory neurotransmitter release. Moreover, recovery from the static symptoms is concurrent with increased presynaptic GABA_B autoreceptor activity and expression on the intact side of compensating chickens. The high degree of synaptic plasticity documented here at the inhibitory terminals contacting the tangential principal cells after UVG underscores that the principal cells function in complex signal integration rather than as simple relay neurons, since synaptic plasticity characterizes the former and is lacking in the latter (Bender and Trussell, 2011). The next step is to identify the source or sources of the GABA_B-receptor labeled inhibitory inputs to the principal cells and determine to what extent their modified synaptic transmission in principal cells alters the remaining components in the vestibular neural circuitry and vestibular reflex behaviors before and after UVG.

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References

- Akbarian S, Grusser OJ, Guldin WO. Corticofugal projections to the vestibular nucleus in squirrel monkeys: further evidence of multiple cortical vestibular fields. *J Comp Neurol*. 1993; 332:89–104. [PubMed: 7685782]
- Akhtar ND, Land PW. Activity-dependent regulation of glutamic acid decarboxylase in the rat barrel cortex: effects of neonatal versus adult sensory deprivation. *J Comp Neurol*. 1991; 307:200–213. [PubMed: 1713230]
- Aldrich EM, Peusner KD. Vestibular compensation after ganglionectomy: ultrastructural study of the tangential vestibular nucleus and behavioral study of the hatchling chick. *J Neurosci Res*. 2002; 67:122–138. [PubMed: 11754088]
- Astori S, Luján R, K hr G. GABA release from cerebellar stellate cells is developmentally regulated by presynaptic GABA (B) receptors in a target-cell-specific manner. *Eur J Neurosci*. 2009; 30:551–559. [PubMed: 19674089]
- Badke MB, Pyle GM, Shea T, Miedaner J. Outcomes in vestibular ablative procedures. *Otol Neurotol*. 2002; 23:504–509. [PubMed: 12170153]
- Bailey SJ, Dhillon A, Woodhall GL, Jones RS. Lamina-specific differences in GABA(B) autoreceptor-mediated regulation of spontaneous GABA release in rat entorhinal cortex. *Neuropharmacology*. 2004; 46:31–42. [PubMed: 14654095]

- Bechterew von W. Ergebnisse der Durchschneidung des N. acusticus nebst Erörterung der Bedeutung der semicirculären Kanäle für das Körpergleichgewicht. *Pflügers Arch Ges Physiol.* 1883; 30:312–347.
- Ben-Ari Y. Excitatory actions of GABA during development: the nature of the nurture. *Nat Rev Neurosci.* 2002; 3:728–739. [PubMed: 12209121]
- Bender KJ, Trussell LO. Synaptic plasticity in inhibitory neurons of the auditory brainstem. *Neuropharmacology.* 2011; 60:774–779. [PubMed: 21185317]
- Beranek M, Idoux E. Reconsidering the role of neuronal intrinsic properties and neuromodulation in vestibular homeostasis. *Front Neurol.* 2012; 3:25. [PubMed: 22403570]
- Bergquist F, Ludwig M, Dutia MB. Role of the commissural inhibitory system in vestibular compensation in the rat. *J Physiol.* 2008; 586:4441–4452. [PubMed: 18635647]
- Bettler B, Tiao JY. Molecular diversity, trafficking and subcellular localization of GABAB receptors. *Pharmacol Ther.* 2006; 110:533–543. [PubMed: 16644017]
- Caillard O. Pre & postsynaptic tuning of action potential timing by spontaneous GABAergic activity. *PLoS One.* 2011; 6:e22322. [PubMed: 21789249]
- Calver AR, Medhurst AD, Robbins MJ, Charles KJ, Evans ML, Harrison DC, Stammers M, Hughes SA, Hervieu G, Couve A, Moss SJ, Middlemiss DN, Pangalos MN. The expression of GABA (B1) and GABA (B2) receptor subunits in the CNS differs from that in peripheral tissues. *Neuroscience.* 2000; 100:155–170. [PubMed: 10996466]
- Capogna M, Gähwiler BH, Thompson SM. Calcium-independent actions of alpha-latrotoxin on spontaneous and evoked synaptic transmission in the hippocampus. *J Neurophysiol.* 1996; 76:3149–3158. [PubMed: 8930262]
- Carter AG, Regehr WG. Quantal events shape cerebellar interneuron firing. *Nat Neurosci.* 2002; 5:1309–1318. [PubMed: 12411959]
- Caspary DM, Ling L, Turner JG, Hughes LF. Inhibitory neurotransmission, plasticity and aging in the mammalian central auditory system. *J Exp Biol.* 2008; 211:1781–1791. [PubMed: 18490394]
- Chung SH, Kim CT, Hawkes R. Compartmentation of GABA b receptor2 expression in the mouse cerebellar cortex. *Cerebellum.* 2008; 7:295–303. [PubMed: 18418671]
- Cossart R, Hirsch JC, Cannon RC, Dinoncourt C, Wheal HV, Ben-Ari Y, Escalapez M, Bernard C. Distribution of spontaneous current along the somato-dendritic axis of rat hippocampus CA1 pyramidal neurons. *Neuroscience.* 2000; 99:593–603. [PubMed: 10974423]
- Couve A, Thomas P, Calver AR, Hirst WD, Pangalos MN, Walsh FS, Smart TG, Moss SJ. Cyclic AMP-dependent protein kinase phosphorylation facilitates GABA(B) receptor-effector coupling. *Nat Neurosci.* 2002; 5:415–424. [PubMed: 11976702]
- Cox RG, Peusner KD. Horseradish peroxidase labeling of the efferent and afferent pathways of the avian tangential vestibular nucleus. *J Comp Neurol.* 1990; 296:324–341. [PubMed: 2358540]
- Dani VS, Chang Q, Maffei A, Turrigiano GG, Jaenisch R, Nelson SB. Reduced cortical activity due to a shift in the balance between excitation and inhibition in a mouse model of Rett Syndrome. *Proc Natl Acad Sci USA.* 2005; 102:12560–12565. [PubMed: 16116096]
- de Waele C, Mhlethaler M, Vidal PP. Neurochemistry of the central vestibular pathways. *Brain Res Brain Res Rev.* 1995; 20:24–46. [PubMed: 7711766]
- Dieringer N. ‘Vestibular compensation’: neural plasticity and its relations to functional recovery after labyrinthine lesions in frogs and other vertebrates. *Prog Neurobiol.* 1995; 46:97–129. [PubMed: 7568917]
- Dieringer N. Activity-related postlesional vestibular reorganization. *Ann N Y Acad Sci.* 2003; 1004:50–60. [PubMed: 14662447]
- Eleore L, Vassias I, Bernat I, Vidal PP, de Waele C. An in situ hybridization and immunofluorescence study of GABAA and GABAB receptors in the vestibular nuclei of the intact and unilateral labyrinthectomized rat. *Exp Brain Res.* 2005; 160:166–179. [PubMed: 15452674]
- Faugier-Grimaud S, Ventre J. Anatomic connections of inferior parietal cortex (area 7) with subcortical structures related to vestibulo-ocular function in a monkey (*Macaca fascicularis*). *J Comp Neurol.* 1989; 280:1–14. [PubMed: 2465325]
- Fox MA, Sanes JR. Synaptotagmin I and II are present in distinct subsets of central synapses. *J Comp Neurol.* 2007; 503:280–296. [PubMed: 17492637]

- Fritschy JM, Sidler C, Parpan F, Gassmann M, Kaupmann K, Bettler B, Benke D. Independent maturation of the GABA(B) receptor subunits GABA(B1) and GABA(B2) during postnatal development in rodent brain. *J Comp Neurol*. 2004; 477:235–252. [PubMed: 15305362]
- Fuchs JL, Salazar E. Effects of whisker trimming on GABA(A) receptor binding in the barrel cortex of developing and adult rats. *J Comp Neurol*. 1998; 395:209–216. [PubMed: 9603373]
- Gallagher JP, Phelan KD, Shinnick-Gallagher P. Modulation of excitatory transmission at the rat medial vestibular nucleus synapse. *Ann N Y Acad Sci*. 1992; 656:630–644. [PubMed: 1350894]
- Galvez T, Duthey B, Kniazeff J, Blahos J, Rovelli G, Bettler B, Prezeau L, Pin JP. Allosteric interactions between GB1 and GB2 subunits are required for optimal GABA(B) receptor function. *EMBO J*. 2001; 20:2152–2159. [PubMed: 11331581]
- Gamkrelidze G, Giaume C, Peusner KD. The differential expression of low-threshold sustained potassium current contributes to the distinct firing patterns in embryonic central vestibular neurons. *J Neurosci*. 1998; 18:1449–1464. [PubMed: 9454854]
- Gao XP, Liu QS, Liu Q, Wong-Riley MT. Excitatory-inhibitory imbalance in hypoglossal neurons during the critical period of postnatal development in the rat. *J Physiol*. 2011; 589:1991–2006. [PubMed: 21486774]
- Geppert M, Archer BT, Sudhof TC. Synaptotagmin II. A novel differentially distributed form of synaptotagmin. *J Biol Chem*. 1991; 266:13548–13552. [PubMed: 1856191]
- Gliddon CM, Darlington CL, Smith PF. GABAergic systems in the vestibular nucleus and their contribution to vestibular compensation. *Prog Neurobiol*. 2005; 75:53–81. [PubMed: 15713530]
- Gottesman-Davis A, Peusner KD. Identification of vestibuloocular projection neurons in the developing chicken medial vestibular nucleus. *J Neurosci Res*. 2010; 88:290–303. [PubMed: 19705454]
- Gross GH. Innervation of the complexus (“hatching”) muscle of the chick. *J Comp Neurol*. 1985; 232:180–189. [PubMed: 3973089]
- Guldin WO, Mirring S, Grasser OJ. Connections from the neocortex to vestibular brain stem nuclei in the common marmoset. *Neuroreport*. 1993; 5:113–116. [PubMed: 8110998]
- Halmagyi GM, Weber KP, Curthoys IS. Vestibular function after acute vestibular neuritis. *Restor Neurol Neurosci*. 2010; 28:37–46. [PubMed: 20086281]
- Han EB, Stevens CF. Development regulates a switch between post- and presynaptic strengthening in response to activity deprivation. *Proc Natl Acad Sci USA*. 2009; 106:10817–10822. [PubMed: 19509338]
- Hendry SH, Jones EG. Reduction in number of immunostained GABAergic neurones in deprived-eye dominance columns of monkey area 17. *Nature*. 1986; 320:750–753. [PubMed: 3703001]
- Hendry SH, Jones EG. Activity-dependent regulation of GABA expression in the visual cortex of adult monkeys. *Neuron*. 1988; 1:701–712. [PubMed: 3272185]
- Heskin-Sweezie R, Titley HK, Baizer JS, Broussard DM. Type B GABA receptors contribute to the restoration of balance during vestibular compensation in mice. *Neuroscience*. 2010; 169:302–314. [PubMed: 20394801]
- Highstein SM, Holstein GR. The anatomy of the vestibular nuclei. *Prog Brain Res*. 2006; 151:157–203. [PubMed: 16221589]
- Holstein GR, Martinelli GP, Cohen B. L-baclofen GABAB binding sites in the medial vestibular nucleus localized by immunocytochemistry. *Brain Res*. 1992; 581:175–180. [PubMed: 1323367]
- Johnston AR, Him A, Dutia MB. Differential regulation of GABA(A) and GABA(B) receptors during vestibular compensation. *Neuroreport*. 2001; 12:597–600. [PubMed: 11234771]
- Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C. GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. *Nature*. 1998; 396:674–679. [PubMed: 9872315]
- Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B. GABA(B)-receptor subtypes assemble into functional heteromeric complexes. *Nature*. 1998a; 396:683–687. [PubMed: 9872317]
- Kaupmann K, Schuler V, Mosbacher J, Bischoff S, Bittiger H, Heid J, Froestl W, Leonhard S, Pfaff T, Karschin A, Bettler B. Human gamma-aminobutyric acid type B receptors are differentially

- expressed and regulate inwardly rectifying K⁺ channels. *Proc Natl Acad Sci USA*. 1998b; 95:14991–14996. [PubMed: 9844003]
- Kuner R, Kohr G, Grunewald S, Eisenhardt G, Bach A, Kornau HC. Role of heteromer formation in GABAB receptor function. *Science*. 1999; 283:74–77. [PubMed: 9872744]
- Kuypers HG, Lawrence DG. Cortical projections to the red nucleus and the brain stem in the Rhesus monkey. *Brain Res*. 1967; 4:151–188. [PubMed: 4961812]
- Lambert FM, Straka H. The frog vestibular system as a model for lesion-induced plasticity: basic neural principles and implications for posture control. *Front Neurol*. 2012 in press.
- Li H, Prince DA. Synaptic activity in chronically injured, epileptogenic sensory-motor neocortex. *J Neurophysiol*. 2002; 88:2–12. [PubMed: 12091528]
- Li DP, Yang Q, Pan HM, Pan HL. Plasticity of pre- and postsynaptic GABAB receptor function in the paraventricular nucleus in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol*. 2008; 295:H807–815. [PubMed: 18567709]
- Llinas, R.; Walton, K. Vestibular compensation: a distributed property of the central nervous system. In: Asanuma, H.; Wilson, VJ., editors. *Integration in the Nervous System*. Tokyo: Igaku-Shoin; 1979. p. 145-166.
- Lujan R, Shigemoto R, Lopez-Bendito G. Glutamate and GABA receptor signalling in the developing brain. *Neuroscience*. 2005; 130:567–580. [PubMed: 15590141]
- Magnusson AK, Eriksson B, Tham R. Effects of the GABA agonists baclofen and THIP on long-term compensation in hemilabyrinthectomised rats. *Brain Res*. 1998; 795:307–311. [PubMed: 9622660]
- Magnusson AK, Lindstrom S, Tham R. GABA(B) receptors contribute to vestibular compensation after unilateral labyrinthectomy in pigmented rats. *Exp Brain Res*. 2000; 134(1):32–41. [PubMed: 11026723]
- Magnusson AK, Ulfendahl M, Tham R. Early compensation of vestibulo-oculomotor symptoms after unilateral vestibular loss in rats is related to GABA(B) receptor function. *Neuroscience*. 2002; 111:625–634. [PubMed: 12031349]
- Margeta-Mitrovic M, Jan YN, Jan LY. A trafficking checkpoint controls GABA(B) receptor heterodimerization. *Neuron*. 2000; 27:97–106. [PubMed: 10939334]
- McKinney RA, Capogna M, Durr R, Gähwiler BH, Thompson SM. Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nat Neurosci*. 1999; 2:44–49. [PubMed: 10195179]
- Mittelstaedt T, Seifert G, Alvarez-Baron E, Steinhauser C, Becker AJ, Schoch S. Differential mRNA expression patterns of the synaptotagmin gene family in the rodent brain. *J Comp Neurol*. 2009; 512:514–528. [PubMed: 19030179]
- Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ. Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *J Neurosci*. 2002; 22:6724–6731. [PubMed: 12151551]
- National Research Council. Guidelines for the care and use of mammals in neuroscience and behavioral research. National Academy Press; Washington, DC: 2003.
- Neher E, Sakaba T. Multiple roles of calcium ions in the regulation of neurotransmitter release. *Neuron*. 2008; 59:861–872. [PubMed: 18817727]
- Ng GY, Clark J, Coulombe N, Ethier N, Hebert TE, Sullivan R, Kargman S, Chateaufneuf A, Tsukamoto N, McDonald T, Whiting P, Mezey E, Johnson MP, Liu Q, Kolakowski LF, Evans JF, Bonner TI, O'Neill GP. Identification of a GABA_B receptor subunit, gb2, required for functional GABAB receptor activity. *J Biol Chem*. 1999; 274:7607–7610. [PubMed: 10075644]
- Owens DF, Kriegstein AR. Is there more to GABA than synaptic inhibition? *Nat Rev Neurosci*. 2002; 3:715–727. [PubMed: 12209120]
- Peusner KD, Morest DK. The neuronal architecture and topography of the nucleus vestibularis tangentialis in the late chick embryo. *Neuroscience*. 1977; 2:189–207. [PubMed: 904774]
- Peusner KD, Shao M, Reddaway R, Hirsch JC. Basic Concepts in Understanding Recovery of Function in Vestibular Reflex Networks during Vestibular Compensation. *Front Neurol*. 2012; 3:17. [PubMed: 22363316]
- Pollack SM, Popratiloff A, Peusner KD. Vestibular ganglionectomy and otolith nerve identification in the hatchling chicken. *J Neurosci Methods*. 2004; 138:149–155. [PubMed: 15325123]

- Popratiloff A, Giaume C, Peusner KD. Developmental change in expression and subcellular localization of two shaker-related potassium channel proteins (Kv1.1 and Kv1.2) in the chick tangential vestibular nucleus. *J Comp Neurol*. 2003; 461:466–482. [PubMed: 12746863]
- Popratiloff A, Wang YX, Narvid J, Petralia RS, Giaume C, Peusner KD. AMPA receptor subunit expression in chick vestibular nucleus neurons. *J Neurosci Res*. 2004; 76:662–677. [PubMed: 15139025]
- Popratiloff A, Peusner KD. GABA and glycine immunolabeling in the chicken tangential nucleus. *Neuroscience*. 2011; 175:328–343. [PubMed: 21129450]
- Precht W, Shimazu H, Markham CH. A mechanism of central compensation of vestibular function following hemilabyrinthectomy. *J Neurophysiol*. 1966; 29:996–1010. [PubMed: 5971666]
- Rosier AM, Arckens L, Demeulemeester H, Orban GA, Eysel UT, Wu YJ, Vandesande F. Effect of sensory deafferentation on immunoreactivity of GABAergic cells and on GABA receptors in the adult cat visual cortex. *J Comp Neurol*. 1995; 359:476–489. [PubMed: 7499542]
- Sara Y, Virmani T, Deák F, Liu X, Kavalali ET. An isolated pool of vesicles recycles at rest and drives spontaneous neurotransmission. *Neuron*. 2005; 45:563–573. [PubMed: 15721242]
- Sato H, Ohkawa T, Uchino Y, Wilson VJ. Excitatory connections between neurons of the central cervical nucleus and vestibular neurons in the cat. *Exp Brain Res*. 1997; 115:381–386. [PubMed: 9262193]
- Segal M. Dendritic spines, synaptic plasticity and neuronal survival: activity shapes dendritic spines to enhance neuronal viability. *Eur J Neurosci*. 2010; 31:2178–2184. [PubMed: 20550565]
- Shao M, Hirsch JC, Giaume C, Peusner KD. Spontaneous synaptic activity is primarily GABAergic in vestibular nucleus neurons of the chick embryo. *J Neurophysiol*. 2003; 90:1182–1192. [PubMed: 12904504]
- Shao M, Hirsch JC, Giaume C, Peusner KD. Spontaneous synaptic activity in chick vestibular nucleus neurons during the perinatal period. *Neuroscience*. 2004; 127:81–90. [PubMed: 15219671]
- Shao M, Popratiloff A, Yi J, Lerner A, Hirsch JC, Peusner KD. Adaptation of chicken vestibular nucleus neurons to unilateral vestibular ganglionectomy. *Neuroscience*. 2009; 161:988–1007. [PubMed: 19375485]
- Shao M, Hirsch JC, Peusner KD. Plasticity of spontaneous excitatory and inhibitory synaptic activity in morphologically defined vestibular nuclei neurons during early vestibular compensation. *J Neurophysiol*. 2012; 107:29–41. [PubMed: 21957228]
- Smith PF, Curthoys IS. Mechanisms of recovery following unilateral labyrinthectomy: a review. *Brain Res Brain Res Rev*. 1989; 14:155–180. [PubMed: 2665890]
- Spiegel EA, Demetriades TD. Die zentrale compensation des labyrinth verlusters. *Pflugers Arch Gesamte Physiol*. 1925; 210:215–222.
- Steen PA, Mason M, Pham L, Lefebvre Y, Hickmott PW. Axonal bias at a representational border in adult rat somatosensory cortex (S1). *J Comp Neurol*. 2007; 500:634–645. [PubMed: 17154268]
- Sutton MA, Taylor AM, Ito HT, Pham A, Schuman EM. Postsynaptic decoding of neural activity: eEF2 as a biochemical sensor coupling miniature synaptic transmission to local protein synthesis. *Neuron*. 2007; 55:648–661. [PubMed: 17698016]
- Suzuki N, Bekkers JM. Inhibitory neurons in the anterior piriform cortex of the mouse: classification using molecular markers. *J Comp Neurol*. 2010; 518:1670–1687. [PubMed: 20235162]
- Thompson SE, Ayman G, Woodhall GL, Jones RS. Depression of glutamate and GABA release by presynaptic GABAB receptors in the entorhinal cortex in normal and chronically epileptic rats. *Neurosignals*. 2006; 15:202–215. [PubMed: 17215590]
- Tremblay E, Ben-Ari Y, Roisin MP. Different GABAB-mediated effects on protein kinase C activity and immunoreactivity in neonatal and adult rat hippocampal slices. *J Neurochem*. 1995; 65:863–870. [PubMed: 7616247]
- Vale C, Sanes DH. The effect of bilateral deafness on excitatory and inhibitory synaptic strength in the inferior colliculus. *Eur J Neurosci*. 2002; 16:2394–2404. [PubMed: 12492434]
- Vibert N, Serafin M, Vidal PP, Mhlethaler M. Effects of baclofen on medial vestibular nucleus neurones in guinea-pig brainstem slices. *Neurosci Lett*. 1995; 183:193–197. [PubMed: 7739792]

- Vibert N, Babalian A, Serafin M, Gasc JP, Muhlethaler M, Vidal PP. Plastic changes underlying vestibular compensation in the guinea-pig persist in isolated, in vitro whole brain preparations. *Neuroscience*. 1999; 93:413–432. [PubMed: 10465424]
- Villemure JF, Adam L, Bevan NT, Gearing K, Chenier S, Bouvier M. Subcellular distribution of GABA(B) receptor homo- and hetero-dimers. *Biochem J*. 2005; 388:47–55. [PubMed: 15617512]
- Wallman J, Velez J, Weinstein B, Green AE. Avian vestibuloocular reflex: adaptive plasticity and developmental changes. *J Neurophysiol*. 1982; 48:952–967. [PubMed: 6982960]
- Wang X, Zhang HM, Chen SR, Pan HL. Altered synaptic input and GABAB receptor function in spinal superficial dorsal horn neurons in rats with diabetic neuropathy. *J Physiol*. 2007; 579:849–861. [PubMed: 17218355]
- White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH. Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature*. 1998; 396:679–682. [PubMed: 9872316]
- Wold JE. The vestibular nuclei in the domestic hen (*Gallus domesticus*): ascending projections to the mesencephalic eye motor nuclei. *J Comp Neurol*. 1978; 179:393–406. [PubMed: 641224]
- Yamanaka T, Him A, Cameron SA, Dutia MB. Rapid compensatory changes in GABA receptor efficacy in rat vestibular neurons after unilateral labyrinthectomy. *J Physiol*. 2000; 523:413–424. [PubMed: 10699085]

Highlights

- Major changes in presynaptic GABA_B receptors occur during vestibular compensation
- GABA_B decreases GABA release in vestibular nuclei neurons after lesions
- GABA_B decreases GABA activity on the intact side of compensating chickens
- Postsynaptic GABA_B receptors are not involved in vestibular compensation

Fig.1.

Confocal images of positive and negative controls for antibody specificity performed on chicken cerebellum (A-D) and/or the tangential nucleus (E). A: Confocal tile image of positive control for guinea pig anti-GABA_BR2 primary antibody showing positive immunolabeling for cerebellar Purkinje cell bodies (arrow) and dendrites in the molecular layer (M). Scale bar, 20 μ m. B₁: Positive control for primary antibody to Syt1 which labeled many terminals in the cerebellar molecular layer (M). B₂: On plotting the line scan from B₁, regions of increased pixel brightness were detected along the line, representing high signal/noise ratio that corresponded to Syt1-labeled terminals. Length of line scan in B₁, 200 μ m. C₁: Positive control for primary antibody to Syt2 which labeled many terminals surrounding the cerebellar Purkinje cell bodies (arrow). C₂: On plotting the line scan from C₁ regions of increased pixel brightness were observed along the line, representing high signal/noise ratio that corresponded to Syt2-labeled terminals. Length of line scan in C₁ 250 μ m. D: Negative control for GABA_BR2 immunolabeling was performed by omitting guinea pig anti-GABA_BR2 primary antibody. D₁: Confocal image of cerebellar Purkinje cells showed no GABA_BR2 signal when captured using the same parameters applied to take experimental images in the study. D₂: Confocal image brightness was boosted so that a line could be drawn through the Purkinje cell bodies. D₃: Line scan through the Purkinje cell bodies in D₂ indicated low GABA_BR2 signal/noise ratio. Length of the line scan in D₁ and D₂ was 200

μm. E: Confocal image demonstrating a cross-over control experiment in which guinea pig anti-GABA_BR2 primary antibody was reacted with secondary anti-mouse IgG, used here to reveal Syt1, Syt2, and MAP2. No GABA_BR2 immunolabeling of principal cell bodies in the tangential nucleus was detected. E₁: Image of a principal cell body lacking GABA_BR2 signal when captured using the same parameters to take experimental images in this study. E₂: Image brightness was boosted to detect the principal cell body so that a line could be drawn through the soma. E₃: Line scan through the principal cell body in E₂ revealed low signal/noise ratio for GABA_BR2 immunolabeling. Length of the line scans in E₁ and E₂ was 100 μm.

Fig. 2.

A: Recording from a principal cell in H7 control during exposure to 10 μ M baclofen for 90 seconds at a holding potential of -65 mV. No postsynaptic current was detected. B-J: High power (63X) confocal images of MAP2 (B, E, H) and GABA_BR2 (C, F, I) immunolabeling in principal cell bodies in the tangential nucleus from controls (B-D), lesion (E-G) and intact (H-J) sides of compensating chickens three days after UVG. Note the increased GABA_BR2 immunolabeling in MAP2-positive principal cell bodies on the intact side of compensating chickens (I, J) compared to controls (C, D). K: Mean pixel brightness of GABA_BR2 immunolabeling (gray scale 0-255) increased significantly in the principal cell body cytoplasm on the intact side of compensating chickens compared to controls. L: Mean pixel brightness of GABA_BR2 immunolabeling was weak in line scans drawn on the cell surface compared to that in the cytoplasm of MAP2-positive principal cell bodies from controls, and did not change in compensating chickens. K, L: Using two different approaches, GABA_BR2 immunolabeling increased in the principal cell body cytoplasm on the intact side of compensating chickens relative to controls (see Experimental Procedures). Scale bar (J), 20 μ m.

Fig. 3.

Decreased mEPSC frequency on exposure to 10 μ M baclofen in principal cells from H7 controls (A) and on the intact side of uncompensated chickens three days after UVG (B). A₁ and A₂, and B₁ and B₂ were taken from the same cells, respectively. C: After baclofen exposure, mEPSC frequency decreased in principal cells in all experimental groups. D: After baclofen exposure, the percentage decrease in mEPSC frequency was significantly greater in principal cells on the intact side of uncompensated chickens compared to controls and the lesion side of compensating and uncompensated chickens three days after UVG.

Fig. 4.

Decreased GABAergic mIPSC frequency on exposure to 10 μ M baclofen in principal cells on the lesion (A) and intact (B) sides of compensating chickens three days after UVG. A₁ and A₂, B₁ and B₂ were taken from the same cells, respectively. C: After baclofen exposure, GABAergic mIPSC frequency decreased in principal cells in all experimental groups. D: After baclofen exposure, the percentage decrease in GABAergic mIPSC frequency was significantly greater in principal cells on the intact side of compensating chickens compared to the lesion side

Fig. 5.

Decreased glycinergic mIPSC frequency on exposure to 10 μ M baclofen in principal cells from H7 controls (A): A₁ and A₂ were taken from the same cell. B: After baclofen exposure, glycinergic mIPSC frequency decreased in principal cells from all experimental groups. C: After baclofen exposure, glycinergic mIPSC frequency decreased significantly only in principal cells on the intact side one and three days after UVG. D: After baclofen exposure, the percentage decrease in glycinergic mIPSC frequency was similar in principal cells from all experimental groups.

Fig. 6.

(A-O) High power (63X) confocal images of Syt1 (A, D, G, J, M) and GABA_BR₂ (B, E, H, K, N) immunolabeling in the tangential nucleus from control (A-C), compensating (D-I) and uncompensated chickens (J-O) three days after UVG. Note the decrease in Syt1-labeled terminals on the lesion (J, L) and intact side (M, O) of uncompensated chickens. P, Q: enlarged images of the tangential nucleus from boxed-region in C and O, respectively. Arrows indicate overlap of Syt-1 and GABA_BR₂. R: Mean Syt1-labeled terminal area in the tangential nucleus decreased significantly on both the lesion and intact side in uncompensated chickens. S: Mean GABA_BR₂ pixel brightness in Syt1-positive terminals of the tangential nucleus increased significantly on the lesion and intact sides from uncompensated chickens compared to controls and compensating chickens. T: Percentage overlap of Syt1-labeled terminal area and GABA_BR₂ increased significantly on the intact side in uncompensated chickens three days after UVG compared to controls. Scale bar in A-O: 30 μm. Scale bar in P and Q: 10 μm.

Fig. 7. (A-O) High power (63X) confocal images of Syt2 (A, D, G, J, M) and GABA_BR2 (B, E, H, K, N) immunolabeling in the tangential nucleus from control (A-C), compensating (D-I) and uncompensated chickens (J-O) three days after UVG. P, Q: Enlarged images of the tangential nucleus from the boxed-in regions in C and O, respectively. Arrows point to the overlap of Syt-1 and GABA_BR2. R: Mean Syt2-labeled terminal area in the tangential nucleus decreased significantly in uncompensated chickens. S: Mean pixel brightness of GABA_BR2 immunolabeling in Syt2-positive terminals in the tangential nucleus increased on the intact side of compensating chickens, and both the lesion and intact sides in uncompensated chickens. T: Percentage overlap of Syt2-labeled terminal area and GABA_BR2 decreased significantly on the lesion side in compensating chickens compared to the intact side and controls. Scale bar in A-O: 30 μ m. Scale bar in P and Q: 10 μ m.

Table 1
Postsynaptic GABA_BR2 immunolabeling in principal cells before and after UVG

	Mean pixel brightness of line scans in the cytoplasm	Mean pixel brightness of line scans on the surface membranes	Ratio of surface/cytoplasm
Control	33.7±2.0 *	21.1±1.5	0.65±0.02
Lesion side compensating chickens	41.2±2.8	25.3±3.3	0.57±0.06
Intact side compensating chickens	45.8±2.4 *	25.5±2.2	0.55±0.03

All data were presented as mean±SEM.

* mean pixel brightness of line scans in the cytoplasm increased significantly on the intact side of the compensating chickens compared to controls.

Table 2
Effect of baclofen on miniature synaptic events in principal cells before and after UVG

		Before baclofen				After baclofen			
		Frequency (Hz)	Amplitude (pA)	Rise time (ms)	Decay time (ms)	Frequency (Hz)	Amplitude (pA)	Rise time (ms)	Decay time (ms)
mEPSCs	Control	1.2±0.4	18.9±2	0.32±0.02	0.9±0.1	0.8±0.2	19.6±1.8	0.32±0.02	0.9±0.1
	Lesion side, one day	3.4±1.0	15.5±1.0	0.35±0.01	1.1±0.1	2.2±0.9	14.8±0.3	0.34±0.02	1.2±0.2
	Intact side, One day	1.0±0.3	13.4±1.4	0.33±0.02	1.5±0.3	0.7±0.3	13.8±1.6	0.33±0.2	1.2±0.2
	Lesion side, compensating	1.5±0.4	15.6±1.3	0.36±0.02	1.7±0.2	1±0.2	14.8±1.3	0.39±0.04	1.8±0.2
	Intact side, compensating	1.3±0.3	16±1.3	0.33±0.22	1.2±0.1	0.9±0.2	15.5±1.2	0.36±0.02	1.2±0.2
	Lesion side, uncompensated	3.1±0.8	18.5±1.9	0.34±0.02	1.2±0.2	2.1±0.6	17.0±1.8	0.33±0.02	1.2±0.1
	Intact side, uncompensated	1.5±0.4	14.2±1.8	0.35±0.03	0.8±0.1	0.8±0.2	13.3±1.2	0.35± 0.03	0.9±0.1
GABAergic mIPSCs	Control	4.2±2.5	22.9±3.5	0.78±0.07	6.5±0.8	2.9±1.7	20.3±3.1	0.78±0.06	6.3±0.7
	Lesion side, one day	6.4±3.6	25.4±3.6	0.92±0.1	6.4±0.8	3.8±2.0	22.8±3.2	1.0±0.13	6.3±0.8
	Intact side, One day	4.1±1.6	25±2.5	0.67±0.06	6.3±1.0	2.4±0.8	20.7±2.6	0.74±0.06	6.5±0.8
	Lesion side, compensating	8.2±3.3	24.8±2.5	0.77±0.05	4.6±0.4	6.8±2.9	22.3±4.3	0.8±0.05	5.6±0.9
	Intact side, compensating	1.0±0.5	20.9±1.1	1.1±0.14	9.2±1.9	0.4±0.2	17.9±1.1	1.1±0.1	8.6±0.8
	Lesion side, uncompensated	4.3±1.9	22.4±3.6	0.84±0.05	7.2±0.7	2.4±1.1	18.6±2.4	0.82±0.09	6.9±0.7
	Intact side, uncompensated	2.2±0.6	21.3±1.8	1.2±0.19	9.6±0.8	1.3±0.4	17.1±1.8	1.2±0.1	9.5±1
Glycinergic * mIPSCs	Control	0.79±0.19	81±14.5	0.78±0.1	11.8±4.2	0.68±0.16	80.1±15.4	0.71±0.05	11.8±3.7
	Lesion side, one day	1.43±0.39	64.8±9.1	0.86±0.05	9.1±2.5	1.09±0.30	63.2±12.9	0.83±0.06	9.3±2.3
	Intact side, one day	0.92±0.30	81.3±8.7	0.96±0.11	14.1±2.9	0.60±0.19	63.2±9.8	1.11 ±0.16	16.4±4.3
	Lesion side, compensating	0.62±0.18	50.5±4.6	0.72±0.05	4±0.6	0.46±0.14	51.3±5.1	0.73±0.07	4.1±0.4
	Intact side, compensating	0.41±0.08	70.6±6.8	0.8±0.08	10.9±2.9	0.36±0.08	63.9±5.3	0.81±0.08	12.4±3.1
	Lesion side, uncompensated	1.20±0.48	55.5±5.2	0.84±0.07	5.1±0.8	1.01±0.36	57.4±7.9	0.83±0.05	5.5±1
	Intact side, uncompensated	0.44±0.13	46.2±8.6	0.99±0.13	23.3±6.9	0.34±0.09	53.1±6.7	1.1±0.22	25.9±7.5

All the data are presented as mean±SEM. No significant change was detected in the kinetics of miniature events after baclofen coexposure in principal cells from all experimental groups.

* Due to the low frequency of glycinergic events, two decimal places are indicated to show significance. Also, rise time is stated using two decimal places due to the small values.

Table 3
Double-labeling of GABA_BR2 and Syt1- or Syt2-terminal labeling in the tangential nucleus before and after UVG

	controls	Lesion side, compensating	Intact side, uncompensated	Lesion side, uncompensated	Intact side, uncompensated
GABA _B R2 and Syt1	Syt1-labeled terminal area in 135x135 μm ² image (μm ²)	721±68.9 ^a	880±79.7	267±43.3 ^a	294±17.7 ^a
	% Syt1-labeled terminal area/total sampled area	3.9 ^a	4.7	1.4 ^a	1.6 ^a
	Mean pixel brightness of GABA _B R2 in Syt1-labeled terminals	56.9±2.9 ^{b, e}	61.4±3.2 ^e	73.2±2.8 ^e	76.1±1.6 ^e
	% of Syt1-labeled terminals overlapping with GABA _B R2	31.7 ^{c, f}	32.7	33.4 ^f	41.4 ^f
GABA _B R2 and Syt2	Syt2-labeled terminal area in 135x135 μm ² image (μm ²)	635±41 ^a	803±69.2	380±84.1 ^a	430±69.0 ^a
	% Syt2-labeled terminal area/total sampled area	3.4 ^a	4.4	2.0 ^a	2.3 ^a
	Mean pixel brightness of GABA _B R2 in Syt2-labeled terminals	44.8±2.2 ^{b, g}	47.8±2.6 ^d	67.5±2.9 ^g	66.9±4.3 ^g
	% of Syt2-labeled terminals overlapping with GABA _B R2	43.1 ^c	29.6 ^d	37.4	37.7

All the data are presented as mean±SEM.

^a Syt1-labeled terminal area and percentage Syt1-labeled terminal area of the total sampled area decreased significantly on the lesion and intact sides in the uncompensated chickens three days after UVG compared to controls.

^b Mean pixel brightness of GABA_BR2 in Syt1-labeled terminals was significantly higher compared to that in Syt2-labeled terminals in controls.

^c Percentage of Syt2-labeled terminal area overlapping with GABA_BR2 was significantly higher compared to that in Syt1-labeled terminals in controls.

^d Mean pixel brightness of GABA_BR2 in Syt2-labeled terminals and percent of Syt2-labeled terminal area overlapping with GABA_BR2 increased significantly on the intact side compared to the lesion side in the compensating chickens.

^e Mean pixel brightness of GABA_BR2 immunolabeling of Syt1-labeled terminals increased significantly on both lesion and intact sides of uncompensated chickens compared to controls, and lesion and intact sides of compensating chickens.

^f Percentage of Syt1-labeled terminal area overlapping with GABA_BR2 increased significantly on the intact side compared to the lesion side of uncompensated chickens and controls.

^g Mean pixel brightness of GABA_BR2 immunolabeling of Syt2-labeled terminal area increased significantly on both lesion and intact sides in uncompensated chickens compared to controls.