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**Spiral ganglion cell degeneration-induced deafness
as a consequence of reduced GATA factor activity**

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Abstract

Zinc-finger transcription factors GATA2 and GATA3 are both expressed in the developing inner ear, while their overlapping versus distinct activities in adult definitive inner ear is not well understood. We demonstrate here that GATA2 and GATA3 are co-expressed in cochlear spiral ganglion cells and redundantly function in the maintenance of spiral ganglion cells and auditory neural circuitry. Notably, *Gata2* and *Gata3* compound heterozygous mutant mice had a diminished number of spiral ganglion cells due to enhanced apoptosis, which resulted in progressive hearing loss. The decrease of spiral ganglion cellularity was associated with lowered expression of neurotrophin receptor TrkC that is an essential factor for spiral ganglion cell survival. We further demonstrate that *Gata2*-null mutants that additionally bear a *Gata2* YAC (yeast artificial chromosome) that counteracts the lethal hematopoietic deficiency due to complete *Gata2* loss nonetheless failed to complement the deficiency in neonatal spiral ganglion neurons. Furthermore, cochlea-specific *Gata2* deletion mice also had fewer spiral ganglion cells and resultant hearing impairment. These results demonstrate that GATA2 and GATA3 redundantly function to maintain spiral ganglion cells and hearing. We propose possible mechanisms underlying hearing loss in human *GATA2* or *GATA3*-related genetic disorders.

Introduction

The mammalian inner ear is a complex and delicate sensory organ, consisting of

cochlea, vestibulum and semicircular canals (Whitfield, 2015). Auditory sensation begins in the cochlea of the inner ear; acoustic information is generated by inner ear hair cells of the organ of Corti and reaches spiral ganglion cells within the cochlea. The spiral ganglion cells are bipolar neurons that receive auditory information from the hair cells and transmit that information to the auditory center in the brain (Rubel, 2002).

Development of the cochlea begins as an ectodermal thickening known as the otic placode in the lateral rhombencephalon, which invaginates to form a rudimentary structure called the otic vesicle. Subsequently, the ventral part of the otic vesicle gives rise to the cochlea at embryonic day (e) e9.5. These developmental processes require intrinsic and extrinsic factors that regulate proliferation and differentiation of the otic vesicle to form the mature inner ear structure (Barald, 2004). A number of transcriptional regulators have been identified that play a role in development of the inner ear (Whitfield, 2015). Among them are GATA family of transcription factors, which all contain two C₄ zinc fingers that serve as its DNA binding domain and recognize the cognate consensus motif (A/T)GATA(A/G) (Yamamoto, 1990; Ko and Engel, 1993). The zinc finger domain of the GATA factors is conserved among the six members (*Gata1~6*) that constitute this multigene family. GATA2 and GATA3 are the only family members expressed in the developing inner ear (George, 1994; Liew, 1997; Lakshmanan, 1998, 1999; Lim, 2000; Lillevali, 2004; Moriguchi, 2006; Haugas, 2010). GATA3 is initially expressed in the otic placode at e8.0, and subsequently GATA2 expression follows in the otic vesicle at e9.5 (Lawoko-Kerali, 2002; Lillevali, 2004). Thus, GATA2 and GATA3 expression overlaps in the otic vesicle in early stage embryos.

A series of studies have elucidated a number of the developmental roles played by GATA3 in the inner ear (Pandolfi, 1995; Lim, 2000, van der Wees, 2004, Lillevali, 2004; Duncan, 2011; Moriguchi, 2006, 2018). In *Gata3*-null embryos, inner ear development is arrested, while only a rudimentary otic vesicle is formed (Lim, 2000; Karis, 2001; Moriguchi, 2006; Duncan, 2011). Recently, we identified inner ear-specific regulatory sequences lying 571 kb 3' to the *Gata3*-gene and designated that as the otic vesicle enhancer (OVE) (Moriguchi, 2018). When OVE-driven Cre recombinase transgenic mice were crossed with homozygous *Gata3* floxed mutants, the *Gata3* gene was specifically deleted in otic vesicle-derived inner ear tissues, and the

resultant *Gata3* conditionally deficient mice exhibited significant developmental defects in the inner ear (Moriguchi, 2018). Interestingly, molecular and clinical evidence has shown that haploinsufficiency in *GATA3* leads to a dominantly inherited condition known as HDR syndrome, which is associated with the clinical triad of hypoparathyroidism, neural sensory deafness and renal defects (Van Esch, 2000). Thus, the evidence from mice and humans underscore a prominent role for GATA3 in specifying normal inner ear development.

GATA2 plays a crucial role in diverse developmental programs including the hematopoietic, neural and urogenital systems (Tsai, 1994, Zhou, 1998, 2000; Minegishi, 2003; Khandekar, 2004, 2007; Craven, 2004; Hoshino, 2008, Nozawa, 2009; Ainoya, 2012; Lim, 2012). Indeed, *Gata2* homozygous null mutant embryos die by e10.5 from a lethal hematopoietic defect (Tsai, 1994; Suzuki, 2006). Haploid deficiency in human *GATA2* leads to the clinical manifestation of Emberger Syndrome, which presents as lymphedema, myelodysplasia or other hematopoietic disorders, including acute myeloid leukemia (Ostergaard, 2011; Kazenwadel, 2012). Notably, a subsequent clinical report showed that more than 75% of Emberger Syndrome patients had accompanying sensorineural hearing loss, suggesting that GATA2 also participates in the program for human inner ear development (Spinner, 2014). However, the underlying etiological mechanism for deafness that accompanies *GATA2* haploinsufficiency is obscure, largely because the early embryonic lethality of *Gata2*-deficient mice has hampered deeper mechanistic analysis of definitive inner ear development.

In the present study, we addressed the precise expression pattern of GATA2 and GATA3 in the developing inner ear, and examined their possibly overlapping functions in the maintenance of hearing ability. We found that GATA2 and GATA3 expression co-localized in the spiral ganglion cells of the cochlea. *Gata2* and *Gata3* compound heterozygous mutant mice exhibited a diminished number of spiral ganglion cells accompanying a severe hearing loss, while either *Gata2* or *Gata3* single heterozygous mutant mice showed only marginal changes in the spiral ganglion. We demonstrate that homozygous *Gata2* null mutants crossed with *Gata2* YAC transgenic mice (which circumvent the *Gata2*^{-/-} embryonic lethal hematopoietic deficiency) also have significantly fewer spiral ganglion cells in the neonatal stage. Finally, cochlea-specific *Gata2*-conditional deletion mice exhibit a similar decrease in the number of spiral

ganglion cells and subsequent hearing impairment. These results for the first time demonstrate that both GATA2 and GATA3 play redundant roles in the maintenance of spiral ganglion cells and auditory function.

Results

GATA2 and GATA3 are co-expressed in the spiral ganglion cells of the cochlea

To examine the detailed expression pattern of GATA2 and GATA3 during inner ear development, we employed *Gata2* GFP knock-in (*Gata2*^{G/+}) and *Gata3 LacZ* knock-in (*Gata3*^{Z/+}) mice, in which GFP or *LacZ* reporter genes were inserted at the translational initiation site of the *Gata2* and *Gata3* genes, respectively (Suzuki, 2006; Hendriks, 1999). In the *Gata3*^{Z/+} embryos, *LacZ* expression was observed in the otic vesicle at e10.5 and continually detected through e12.5 (arrow heads in Fig. 1A and 1B). In the dissected inner ear tissue of e14.5 *Gata3*^{Z/+} embryos, *LacZ* expression was observed in the three main regions of the inner ear [i.e. in the cochlea (Co), vestibules (Ve), and semicircular canals (SC); Fig. 1C]. In the *Gata2*^{G/+} embryos, GFP expression was distributed in a similar pattern to *LacZ* expression in the *Gata3*^{Z/+} embryos. Robust GFP expression was detected in the otic vesicle of *Gata2*^{G/+} embryos at e10.5 and e12.5 stages (white arrowheads in Fig. 1D and 1E). In the e14.5 embryos, GFP expression was detected in the cochlea, vestibules, and semicircular canal similarly to the *Gata3*^{Z/+} embryos (Fig. 1F). Axial sections of the e14.5 cochlea showed robust GFP fluorescence specifically in the spiral ganglion cells and endothelial cells in the *stria vascularis*, while hair cells in the organ of Corti showed only weak GFP fluorescence (Fig. 1G and 1H).

To histologically assess the expression pattern of *Gata2* and *Gata3* in the cochlea, we crossbred the *Gata2*^{G/+} with *Gata3*^{Z/+} mice and generated *Gata2*^{G/+}::*Gata3*^{Z/+} compound heterozygous mutant mice. Thereafter, we examined the GFP and *LacZ* expression by anti-GFP immunohistochemistry and X-gal staining in the transverse section of the cochlear duct at postnatal day 3 (P3). We found that GFP and *LacZ* were both robustly expressed in the spiral ganglion and organ of Corti (arrows and arrowheads in Fig. 2A and 2D). Detailed analysis at higher magnification demonstrated that the GFP and *LacZ* expression highly co-localized in the spiral ganglion cells in adjacent serial sections (arrowheads in Fig. 2B and 2E). Double staining for GFP and *LacZ* in the same sections clearly showed co-localization of the GFP and X-gal signals in the spiral ganglion (Fig.

2G and 2H). In the organ of Corti, the *LacZ* (i.e., *Gata3* expression) was detected both in the outer and inner hair cells (Fig. 2C and 2I). In contrast, GFP (i.e., *Gata2* expression) was modestly expressed, and only in the outer hair cells (Fig. 2F and 2I). Given the highly overlapping expression of GATA2 and GATA3 in the spiral ganglion cells, we anticipated that GATA2 and GATA3 might contribute to the development and maintenance of spiral ganglion cells in a partially redundant manner.

Diminished spiral ganglion cell numbers in $Gata2^{G/+}::Gata3^{Z/+}$ compound mutant mice

To address the possible functional contributions of GATA2 and GATA3 in the spiral ganglion cells, we first conducted histological analyses by hematoxylin and eosin (HE) examination of $Gata2^{G/+}::Gata3^{Z/+}$ compound heterozygous cochlea sections. We found that the $Gata2^{G/+}::Gata3^{Z/+}$ mice exhibited atrophic changes in the spiral ganglion with decreased cellularity 10 weeks postnatally in comparison with age-matched wild-type mice (compare Fig. 3, A-b to A-k). Quantitative analysis clearly showed that the number of remaining spiral ganglion cells in the $Gata2^{G/+}::Gata3^{Z/+}$ mice was significantly fewer than that of age-matched wild-type mice at 10 weeks of age (Fig. 3B). Individual $Gata2^{G/+}$ or $Gata3^{Z/+}$ heterozygous mutant mice did not exhibit a similar decrease in spiral ganglion cells at the same age (Fig. 3A-e and A-h, and Fig. 3B). Decreased cellularity in spiral ganglia was further exaggerated in 20 week-old $Gata2^{G/+}::Gata3^{Z/+}$ mice (Fig. 3A-l). Quantitative analysis showed that the most significant decrease in the number of spiral ganglion cells was found in the $Gata2^{G/+}::Gata3^{Z/+}$ mice at week 20 (Fig. 3B). $Gata3^{Z/+}$ single heterozygous mutants showed modestly decreased numbers of spiral ganglion cells at 20 weeks of age (Fig. 3A-i and 3B). Despite the pronounced loss in the number of inner ear neurons, overall cochlear architecture was maintained in each genotype of mutant mice at 10 weeks and 20 weeks of age (Fig. 3A-a, d, g, j and data not shown).

Increased ABR threshold in $Gata2^{G/+}::Gata3^{Z/+}$ compound mutant mice

Given the significant decrease in the spiral ganglion cell population in the compound *Gata* mutants, we next subjected adult $Gata2^{G/+}::Gata3^{Z/+}$ mice to functional hearing tests by click-evoked ABR (Auditory Brain-stem Response) at 10 and 20 weeks of age

(Fig. 3C). Interestingly, we found that the ABR threshold was significantly increased in the 10 week old *Gata2*^{G/+}::*Gata3*^{Z/+} mice in comparison to age-matched wild-type mice (80 ± 7 dB vs. 38 ± 5 dB, respectively) (compare black and white bars in Fig. 3C). In comparison, *Gata2*^{G/+} or *Gata3*^{Z/+} heterozygous mutant mice showed normal ABR thresholds at the 10 weeks of age (Fig. 3C). Hearing impairment in the *Gata2*^{G/+}::*Gata3*^{Z/+} mice became progressively exaggerated, as their ABR threshold further increased by 20 weeks of age (Fig. 3C). Heterozygous *Gata3*^{Z/+} mice also showed a slight increase in the ABR threshold (70 ± 10 dB) by 20 weeks of age, while *Gata2*^{G/+} mutant animals remained unaffected. Thus, these results indicate that the simultaneous haploid deficiency in both *Gata2* and *Gata3* synergistically or additively diminishes hearing ability.

Increased apoptosis in the spiral ganglion of *Gata2*^{G/+}::*Gata3*^{Z/+} mice

To address cellular mechanisms underlying the decreased number of spiral ganglion cells in the *Gata2*^{G/+}::*Gata3*^{Z/+} mice, axial sections of cochlea were subjected to TUNEL analysis prior to degeneration at neonatal stage. As anticipated, TUNEL-positive cells were found in the spiral ganglion of the *Gata2*^{G/+}::*Gata3*^{Z/+} mice (Fig. 4D). In contrast, no TUNEL-positive cells were detected in the spiral ganglia of wild-type and *Gata2*^{G/+} littermates (Fig. 4A and B). Quantification showed a statistically significant increase of TUNEL-positive cells in the *Gata2*^{G/+}::*Gata3*^{Z/+} compound mutant mice (Fig. 4E). *Gata3*^{Z/+} mice also showed a smaller increase in apoptosis, which is in agreement with the modest decrease of spiral ganglion cells at 20 weeks of age (Fig. 4C and E). These results suggest that the enhanced apoptosis of spiral ganglion cells led to the loss of cellularity and the subsequent hearing impairment in *Gata2*^{G/+}::*Gata3*^{Z/+} mice.

TrkC expression is markedly reduced in the *Gata2*^{G/+}::*Gata3*^{Z/+} neonates

Neurotrophin signaling by neurotrophin-3 (NT3) through the TrkC receptor and brain-derived neurotrophic factor (BDNF) via the TrkB receptor plays a role for the development and maintenance of spiral ganglion cells (Fritsch, 2004). Expression of TrkB and TrkC in the inner ear spiral ganglion cells depends on NeuroD, a basic helix-loop-helix (bHLH) transcription factor, and thus NeuroD-deficient mice show a severe reduction of spiral ganglion cells (Kim, 2001). Considering these observations,

we addressed the expression profile of these inner ear-affiliated genes to probe possible molecular mechanisms underlying spiral ganglion cell degeneration in the *Gata2*^{G/+}::*Gata3*^{Z/+} mice. To this end, we first examined mRNA expression of these genes after dissecting the spiral ganglion region of cochlea. We found that expression of TrkC was most significantly diminished in the *Gata2*^{G/+}::*Gata3*^{Z/+} mice compared to the wild-type mice (Fig. 5A), while that of TrkB appeared to increase, but without statistical significance (Fig. 5A). The expression of the neurotrophin genes, Bdnf and NT3, were slightly decreased but again without statistical significance, and NeuroD abundance was unaffected (Fig. 5A).

Given the decrease of TrkC expression, we next conducted immunohistochemical analysis for TrkC protein expression. We found that TrkC immunoreactivity was dramatically diminished in the spiral ganglion cells of *Gata2*^{G/+}::*Gata3*^{Z/+} mice, while wild-type mice abundantly expressed TrkC protein in the spiral ganglion cells (Fig. 5B). These results suggest that simultaneous heterozygous deletion of *Gata2* and *Gata3* led to the observed significant reduction of TrkC mRNA and protein in cochlear spiral ganglion cells. We suggest that the decreased level of TrkC expression may account for the enhanced degeneration of spiral ganglion cells.

GATA2 is required for maintenance of spiral ganglion cells

The early embryonic lethal anemia of GATA2-deficient mice obscures any developmental functions of GATA2 at later stages (Tsai, 1994; Suzuki, 2006). To overcome this difficulty, we have previously generated transgenic mouse lines carrying a 271-kb *Gata2* Yeast Artificial Chromosome that contains the entire *Gata2* structural gene plus extended regulatory sequences which direct *Gata2* expression in the hematopoietic system (Tg^{G2YAC}) (Zhou, 1998). We demonstrated that Tg^{G2YAC} mice, when bred into the *Gata2*-null mutant background, rescued the embryonic lethality and allowed them to survive until weaning (Zhou, 1998). However, even this 271 kb YAC lacks regulatory sequences required for otic vesicle-specific *Gata2* expression (Zhou, 1998). We thus addressed whether the Tg^{G2YAC}-rescued *Gata2* null-deficient neonates (*Gata2*^{G/G}::Tg^{G2YAC}) have any developmental defects in the inner ear at the neonatal stage. We found that *Gata2*^{G/G}::Tg^{G2YAC} neonates suffered from a significant decrease of spiral ganglion cell numbers in comparison with the *Gata2*^{G/+} control littermates (Fig.

6B, C, E and F). Quantification confirmed the statistically significant decrease of spiral ganglion cells in the *Gata2*^{G/G::Tg^{G2YAC} mice in comparison with the *Gata2*^{G/+} control (Fig. 6G). The GFP-expression pattern in the dissected cochlea of *Gata2*^{G/G::Tg^{G2YAC} mice was maintained in comparison with the *Gata2*^{G/+} control cochlea, excluding architectural damage (Fig. 6A and D). Hair cells of the organ of Corti in the *Gata2*^{G/G::Tg^{G2YAC} mice were hardly affected (data not shown). These results indicate that GATA2 activity is essential for the development and maintenance of spiral ganglion cells.}}}

Inner ear-specific conditional Gata2-deficient mice have hearing loss

Gata2^{G/G::Tg^{G2YAC} mice succumb to urinary tract anomalies around weaning (Zhou, 1998), because the 271-kb *Gata2* YAC lacks urogenital primordia-specific regulatory sequences located far 3' to the *Gata2* structural gene beyond the YAC boundaries (Khandekar, 2004). Therefore to investigate the consequences of inner ear-specific *Gata2* loss of function at the adult stage, we employed a *Gata2* floxed allele (*Gata2*^f). For inner-ear specific *Gata2* deletion, we crossed a newly developed Cre deleter strain of mice (Tg^{OVE-Cre}) in which Cre recombinase is expressed in the cochlea under the regulation of the otic vesicle-specific enhancer of the mouse *Gata3* gene (Moriguchi, 2018). We confirmed that the OVE-Cre mice labeled the spiral ganglion cells and the organ of Corti, when crossed with ROSA26 R26R floxed *LacZ* reporter mice (Fig. 7A). The *Gata2*^{f/f::Tg^{OVE-Cre} (G2CKO) mice grew to adulthood with grossly normal appearance, and at the expected Mendelian frequency (Table. 1). Upon histological analysis, we found that the G2CKO mice showed decreased spiral ganglion cell numbers compared with age-matched control mice at 20 weeks of age (compare Fig. 7B and C). Quantification of G2CKO and control mice confirmed a statistically significant decrease in spiral ganglion cells in the G2CKO mice (Fig. 7D). Meanwhile, hair cells in the organ of Corti were maintained in the G2CKO mice, indicating that GATA2 is dispensable for the hair cell development (compare Fig. 7E and F). Given these observations, we subjected the G2CKO to ABR analysis evoked by pure tone bursts (4, 8 or 16 kHz). Consistent with the decreased number of spiral ganglion cells, the G2CKO mice exhibited diminished hearing (Fig. 7G). We next examined mRNA expression profile after dissecting the spiral ganglion region of the G2CKO mice. We}}

found that *Gata2* mRNA was decreased to 24.9% in the G2CKO mice compared to that in the wild-type control mice (Fig. 7H), indicating that the *Gata2* floxed allele was deleted in the cochlea tissues. Notably, TrkC expression was diminished in the G2CKO mice compared to the wild-type mice (Fig. 7H), suggesting that TrkC expression is under the regulatory influence of GATA2 in the spiral ganglion. We also found that GATA3 mRNA expression level was rarely affected in the G2CKO mice (Fig. 7H), implying that GATA2 might be dispensable for GATA3 expression in the spiral ganglion cells. These results clearly indicate that GATA2 plays a functional role in the maintenance of spiral ganglion cells and hearing ability.

Discussion

The mammalian inner ear contains sophisticated mechanosensory elements and a series of highly specialized neuronal connections that have evolved for the transduction of mechanical energy to electrical impulses in the cochlear and vestibular nerves. In the auditory system, GATA3 has been recognized to play a role during developmental stages as well as in the adult inner ear, while insight into possible physiological functions and expression of GATA2 particularly in the definitive inner ear has not been defined. We and other groups demonstrated that GATA2 expression shows a similar pattern to that of GATA3 in the early embryonic period (Lillevali, 2004). Subsequently in the postnatal stage, we found that GATA2 and GATA3 are both highly co-expressed in cochlear spiral ganglion cells. In agreement with this overlapping expression pattern, *Gata2*^{G/+}::*Gata3*^{Z/+} compound heterozygous mutant mice showed more severely diminished spiral ganglion cell number than did either *Gata2* or *Gata3* single heterozygous mutant. Spiral ganglion neurons are responsible for the precise connections between hair cells and target neurons in the auditory brainstem (Rubel, 2002). Consequently, *Gata2*^{G/+}::*Gata3*^{Z/+} compound heterozygous mutants showed more severe hearing loss than either the *Gata3*^{Z/+} or *Gata2*^{G/+} heterozygous deficient mice. These results taken together indicate that GATA2 and GATA3 function at least partially redundantly in the maintenance of spiral ganglion cells, and therefore that the gene dosage of *Gata2* and *Gata3* is crucial for the survival of spiral ganglion cells and the maintenance of auditory circuitry.

Signaling by neurotrophin-3 (NT3) through the TrkC receptor or brain-derived neurotrophic factor (Bdnf) via the TrkB receptor support the inner ear sensory neurons

and regulate development of the acoustic neural circuit (Fritzsche, 2004). From early developmental stages into adulthood, NT3 and Bdnf are both expressed in the hair cells in the organ of Corti, while their respective receptors, i.e., TrkB and TrkC are expressed in the developing spiral ganglion cells (Fritzsche, 2004). Consistent with this expression pattern, development of the spiral ganglion cells critically depends on neurotrophin signaling through the Trk receptors. It has been shown that mice deficient for NT3 or TrkC lose the vast majority of spiral ganglion cells in the cochlea (Fritzsche, 1997). In mice lacking either Bdnf or TrkB, the population of spiral ganglion neurons is reduced (Schimmang, 2003). In the present study, we demonstrated that TrkC expression was significantly diminished in the spiral ganglia of *Gata2^{G/+}::Gata3^{Z/+}* as well as G2CKO mice in comparison with the wild type controls, which likely accounts for the loss of spiral ganglion cells in these mutant mice. Notably, TrkB expression tended to be higher in the *Gata2^{G/+}::Gata3^{Z/+}* mice, albeit below statistical significance. TrkB and TrkC, are closely related proteins, which have been reported to redundantly function for neuronal cell survival in the brain (Minichiello, 1996). Therefore, the increase of TrkB in the *Gata2^{G/+}::Gata3^{Z/+}* compound mutant mice might partially compensate for the decrease of TrkC in the spiral ganglion cells. Collectively, the data suggest that TrkC expression could be under either the direct or indirect downstream regulatory influence of GATA2 and GATA3 in the inner ear. In support of this speculation, multiple peaks of GATA2 and GATA3 binding can be detected in nucleotide sequences flanking the TrkC locus in neuroblastoma cell lines (data not shown; UCSF Genome browser. <https://genome.ucsc.edu/>), and therefore we are currently investigating the regulatory activity of these candidate TrkC enhancers.

There have been a series of reports describing regulatory relationships between *Gata2* and *Gata3* in the nervous system. In early embryonic neural development, GATA2 expression precedes that of GATA3, and GATA2 is necessary for GATA3 induction in the chick and mouse central nervous system (Nardelli, 1999; Pata, 1999; Nozawa, 2009). These reports indicate that GATA2 likely functions upstream of GATA3 in the developing central nervous system. In the auditory system, GATA3 governs the developmental program for inner ear morphogenesis. *Gata3*-deficiency leads to an early developmental arrest of the otic vesicle, which is accompanied by decreased GATA2 expression (Karis, 2001; Duncan, 2011; Lillevali, 2004). In contrast, virtually normal

otic vesicle formation occurs in the *Gata2*-null mutant embryos before succumbing to hematopoietic-deficient lethality at e10.5, in which GATA3 expression sustains (Lillevali, 2004). Thus, these data suggest that GATA3 may function hierarchically upstream of GATA2 in early inner ear development.

In the adult inner ear, as we demonstrated here, GATA3 expression domain in the organ of Corti was broader than that of GATA2. The loss of GATA3 elicits a more prominent defect than the loss of GATA2 for the maintenance of spiral ganglion cells. Therefore, GATA3 appears to play a dominant role and possibly acts upstream of GATA2 in the adult inner ear. Consistent with this notion, GATA3 mRNA expression was rarely affected in the G2CKO mice, indicating that GATA2 is dispensable for the GATA3 expression in the inner ear. These results indicate that hierarchical relationship between *Gata2* and *Gata3* in the auditory system is different, and perhaps reversed, from that observed in the developing central nervous system. The complex regulatory relationship between *Gata2* and *Gata3* expression probably depends on the context in which the two factors are encountered during development, and appear to differ from organ to organ.

It has been reported that *Gata2* null-deficient embryos rarely exhibit phenotypic abnormalities early in inner ear development before lethality is encountered by around e10.5. However, the mid-gestational lethality of the *Gata2* null mutant embryos has obscured GATA2 function for the subsequent development and maintenance of the inner ear tissues in the later embryonic and adult stages. An inner ear-specific *Gata2* conditional deletion study employing a *Foxg1*-Cre deleter mouse strain (Hebert and McConnell, 2000; Haugas, 2010) has been investigated previously. However, *Foxg1*-Cre mice induced an unexpectedly broad recombination pattern, and therefore employing that less specific cre deleter mouse model led to global edema and local hemorrhages by e18.5 (Haugas, 2010). Thus, precise GATA2 function in the inner ear during postnatal periods could not be clarified. In this regard, our YAC-rescued *Gata2*-null-deficient mice survived perinatally and exhibited a decrease in the number of spiral ganglion cells as a consequence of *Gata2* loss. Furthermore, the *Gata2* conditional mutants crossed with the newly developed OVE-Cre mice survived to adulthood and had an increased ABR threshold. Both of these new mouse models have clearly been useful in elucidating GATA2 function for inner ear development and

postnatal hearing ability and to clarify the molecular etiology underlying the sensorineural deafness due to GATA2 haploinsufficiency.

Hearing loss is the most common sensory disorder in humans, as congenital hearing loss occurs in approximately 1 affected child out of every 650 newborns (Mehl and Thomson, 2002). A series of studies have described that a progressive loss of the cochlear spiral ganglion cells are very frequently associated with a number of syndromic hearing loss phenotypes in human (Shibata, 2011; Wang, 2018). In this context, it would be of significant interest to ask whether the hearing loss in either *GATA2* or *GATA3* haploinsufficient humans is associated with degenerative loss of spiral ganglion cells, as we demonstrated here in mouse models. Further investigation into the physiological function of GATA2 and GATA3 in the auditory system should provide additional insight into the etiological basis underlying sensorineural deafness and may lead to possible therapeutic approaches for this common sensory disorder.

Experimental procedures

Germ line mutant mice

The *Gata3* *nlacZ* knock-in (*Gata3^Z*) null mutant, *Gata2*-Green Fluorescent Protein (GFP) knock-in (*Gata2^G*) and *Gata2*-floxed alleles (*Gata2^f*) have been described previously (van Doornick, 1999; Hendriks, 1999; Minegishi, 2003; Suzuki, 2006; Lim, 2012). The *Gata2* d16YAC transgenic line (Tg^{G2YAC}), which contains 250 kb of genetic information (from approximately -150 to +80 kb of the *Gata2* locus) was described previously (Zhou, 1998). The generation and characterization of the *Gata3* otic vesicle specific enhancer (OVE)-driven Cre transgenic mice (Tg^{OVE-Cre}) have been reported recently (Moriguchi, 2018). All mice were handled according to the regulations of the standards for use of laboratory animals at Tohoku Medical and Pharmaceutical University, Tohoku University, and the University of Michigan. All animal procedures follow guidelines established for the proper conduct of animal experiments from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

Auditory Brainstem Response (ABR) testing

ABR testing was conducted basically as previously described (Hoshino, 2011). Briefly, wild-type (Wt), *Gata2^{G/+}*, *Gata3^{Z/+}*, *Gata2^{G/+}::Gata3^{Z/+}* compound heterozygous and *Gata2^{f/f}::Tg^{OVE-Cre}* mice, ranging from 10 to 20 weeks old, were subjected to ABR tests

in a soundproof room under anesthesia with intraperitoneal injection of Na⁺pentobarbital (20 mg/kg) (Nembutal; Abbott Laboratories, Chicago, IL). The ABR responses were evoked using bursts of pure tones (4, 8 or 16 kHz) or click sounds, and were recorded with a Synax 1200 system (NEC, Tokyo, Japan). The ABR threshold was determined with increment or decrement of sound pressure in 5 dB steps.

Histological analysis and cell count

Adult mice were anesthetized with pentobarbital sodium (20 mg/kg) and were transcardially perfused with cold phosphate-buffered saline (PBS: 0.1 M, pH 7.4) followed by perfusion with 4% paraformaldehyde (PFA). Mouse tissues were post fixed in 4% paraformaldehyde (PFA) at 4° C overnight. Thereafter, the cochleas were decalcified using 8% EDTA solution and processed for paraffin embedding. Serial paraffin sections (7 μm) were stained with hematoxylin and eosin (HE), and total spiral ganglion neurons in the 1st turn of the cochlea were counted in every fourth section throughout the specimen.

Immunohistochemistry, X-gal staining and TUNEL assay

Gata2^{G/+} embryos (at e10.5, 12.5 and 14.5 dpc) were examined by fluorescence microscopy (Leica Microsystems, Wetzlar, German). Whole embryo X-gal staining were performed as described previously (Lakshmanan, 1998). The *Gata2*^{G/+}::*Gata3*^{Z/+} cochleae were dissected from P3 mice, fixed in 4% PFA and subjected to X-gal staining and immunohistochemistry (IHC) with rabbit anti-GFP antibody (diluted 1:1000; Molecular Probes, Eugene, OR). Apoptotic cells were detected by TUNEL (TdT-mediated dUTP nick end labeling) assay on 10 μm tissue cryosections according to the manufacture's instruction (in situ apoptosis detection kit, TAKARA, Osaka). Methyl Green solution was used for the counterstaining.

RT-PCR analysis using central core region of the cochlea

In order to dissect the presumptive spiral ganglion region, temporal bones of new born mice were removed quickly and dissected in ice-cold PBS (pH 7.4). The spiral ganglion region was dissected from the central core of the cochlea in the temporal bone, which

was subsequently subjected to total RNA isolation using ISOGEN (Nippon-Gene, Osaka, Japan). The RNA sample was subjected to quantitative RT-PCR (RT-qPCR) as described previously (Hoshino, 2011). Primers used for the RT-qPCR analysis are listed in Table 2.

Statistical analyses

All the data are presented as means \pm SD. Statistical analyses were performed using the unpaired Student *t*-test (two tailed). Any test resulting in a *p* value of less than 0.05 was considered significant.

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Figure Legends

Fig. 1. Expression of GATA2 and GATA3 during the inner ear development. (A-B) *Gata3^{Z/+}* embryos show *LacZ* expression in the otic vesicle (arrowheads) at e10.5 and e12.5 dpc. (C) *LacZ* expression was detected in the cochlea (Co), vestibules (Ve), and semicircular canals (SC) in the dissected inner ear tissue of e14.5 *Gata3^{Z/+}* embryo. (D-F) GFP-fluorescence was detected in the otic vesicle (arrowhead) of *Gata3^{G/+}* embryos at e10.5 and e12.5. Inner ear tissue of the e14.5 *Gata2^{G/+}* embryos showed GFP fluorescence in the cochlea, vestibule, and semicircular canals. (G) An axial section of the cochlea of an e14.5 *Gata3^{G/+}* embryo showed robust GFP expression in the spiral ganglion (SG, dotted rectangle) and stria vascularis (SV, arrowheads), while organ of Corti (arrow) showed only weak GFP fluorescence. (H) Higher magnification of the spiral ganglion showed a collection of GFP-positive cells. Scale bars; 40µm (G), 10µm (H).

Fig. 2. GATA2 and GATA3 are co-expressed in the spiral ganglion cells in the cochlea. In the cochlea of postnatal day 3 (P3) *Gata2^{G/+}::Gata3^{Z/+}* mice, GFP-immunoreactivity (brown) and Xgal-staining signals (blue) were detected in the spiral ganglion (SG) and organ of Corti (Co) (A, D and G). At higher magnification, the GFP- and *LacZ*-expression was colocalized in the vast majority of the spiral ganglion cells (arrowheads in B, E and H). (C, F and I) In the organ of Corti, robust *LacZ*-expression was detected in the both outer hair cells (OHC) and inner hair cells (IHC) (C), while modest GFP-expression was detected only in the OHC. Scale bars: (A) and (D); 500 µm. (B) and (E); 200 µm. (C) and (F); 100 µm. (G); 200 µm. (H); 20 µm. (I); 40 µm.

Fig. 3 *Gata2^{G/+}::Gata3^{Z/+}* compound heterozygous mutant mice show progressive spiral ganglion cell loss and hearing impairment with age. (A) Axial sections of the cochlea in each genotype of mice show comparable gross architecture of the cochlear canal at 10 weeks of age (a, d, g and j). *Gata2^{G/+}::Gata3^{Z/+}* mice showed a

progressively diminished number of spiral ganglion cells at 10 and 20 weeks of age (k and l) in comparison with the age-matched wild-type control (b and c). *Gata3^{Z/+}* heterozygous mutant mice had a modest reduction in the spiral ganglion cell population at 20 weeks of age (i). *Gata2^{G/+}* (10 weeks and 20 weeks) and *Gata3^{Z/+}* (10 weeks) mice appeared to have normal cellularity in the spiral ganglion (e, f and h). Scale bars; 200 μ m. (B) Quantitative analyses of the number of residual spiral ganglion cells per cochlea at postnatal day 1 or after 10 weeks or 20 weeks of age. *Gata2^{G/+}::Gata3^{Z/+}* mice had a progressive decrease of the spiral ganglion cell population at 10 and 20 weeks of age. *Gata3^{Z/+}* heterozygous mutant mice showed a modest decrease in spiral ganglion cell number at 20 weeks of age. (C) Click-evoked ABR thresholds of each genotype of mice at 10 and 20 weeks of age. ABR thresholds of *Gata2^{G/+}::Gata3^{Z/+}* mice at 10 weeks of age were significantly more elevated than those in the age-matched wild-type mice. The age-matched *Gata2^{G/+}* or *Gata3^{Z/+}* heterozygous mutants showed normal ABR threshold at 10 weeks of age. ABR thresholds of *Gata2^{G/+}::Gata3^{Z/+}* mice was even more elevated in the 20 week old animals. *Gata3^{Z/+}* heterozygous mutant mice also showed increased ABR thresholds at 20 weeks of age. Data are presented as mean \pm SD. The statistical significance of differences are indicated (* $P<0.05$, ** $P<0.001$; Student's *t* test). More than five mice in each group were subjected to statistical analysis.

Fig. 4. Enhanced apoptosis in the spiral ganglion of *Gata2^{G/+}::Gata3^{Z/+}* mice. A number of TUNEL-positive cells were found in the spiral ganglion of *Gata2^{G/+}::Gata3^{Z/+}* mice (D). In contrast, no TUNEL-positive cells were detected in the spiral ganglia of wild-type or *Gata2^{G/+}* mice (A and B). *Gata3^{Z/+}* had a modest increase in apoptotic cells in the spiral ganglion (C). Scale bars; 10 μ m. (E) Percentage of TUNEL-positive cells among the total spiral ganglion cells enumerated in five sections of each genotype of mice. Data represent mean \pm SD.

Fig. 5. mRNA expression of neurotrophin signaling-related genes. (A) TrkC expression is markedly reduced in the *Gata2^{G/+}::Gata3^{Z/+}* compound mutant mice compared to wild-type control mice. TrkB tended to be higher in the *Gata2^{G/+}::Gata3^{Z/+}* mice, albeit below statistical significance. Bdnf and NT3 levels

were not significantly changed, and NeuroD was also unaffected. The statistical significance of differences are indicated (** $P < 0.01$; Student's *t*-test). (B) TrkC immunoreactivity was markedly reduced in the spiral ganglia of the *Gata2*^{G/+}::*Gata3*^{Z/+} mice (right), whereas wild-type mice abundantly expressed TrkC (left). Scale bars; 20 μ m

Fig. 6. Tg^{G2YAC}-rescued *Gata2* null-deficient neonates showed diminished numbers of spiral ganglion cells. No gross abnormality was found in the GFP-expression pattern in the cochlea of *Gata2*^{G/G}::Tg^{G2YAC} mice in comparison with the *Gata2*^{G/+} cochlea (compare A and D). *Gata2*^{G/G}::Tg^{G2YAC} mice showed a significant reduction of spiral ganglion cells in comparison with the *Gata2*^{G/+} control mice by HE-staining comparison (B, C E and F). Scale bars represent 10 μ m. (G) Number of the spiral ganglion cells in the *Gata2*^{G/G}::Tg^{G2YAC} mice was statistically significantly lower than that of the *Gata2*^{G/+} control mice. More than five mice of each genotype were subjected to the analysis.

Fig. 7. Hearing loss in the inner ear-specific *Gata2*-deficient (G2CKO) mice. (A) Tg^{OVE-Cre} mice crossed with ROSA26 floxed *LacZ* (R26R) reporter mice showed *lacZ* expression in the spiral ganglion cells (arrowhead) and organ of Corti (arrow). (B and C) *Gata2*^{f/f}::Tg^{OVE-Cre} (G2CKO) mice showed diminished spiral ganglion cell numbers at 20 weeks of age in comparison to age matched wild-type control mice. (D) Quantification of the spiral ganglion cell number confirmed a statistically significant decrease in the G2CKO mice (n=10) compared with controls (n=14). (E and F) The hair cell population in the organ of Corti in the G2CKO mice (F) was comparable to that in the control (E). (G) Pure tone bursts (4, 8 or 16 kHz)-evoked ABR thresholds in mutant (CKO) or wild type (control) mice at 20 weeks of age. The ABR thresholds of G2CKO mice were significantly more elevated than those in the age-matched wild-type mice. (H) *Gata2* mRNA is reduced to 24.9% in the G2CKO mice compared to that in the wild-type control mice. Similarly, TrkC expression is reduced in the G2CKO mice. GATA3 mRNA expression level is not significantly different between the the G2CKO and control mice. The statistical significance of

differences are indicated (* $P < 0.05$, ** $P < 0.01$; Student's *t*-test). The data represent the mean \pm SD (n = 6-8). Scale bars: (A)-(C), 200 μ m; (E)-(F), 50 μ m.

Table 1. Genotyping distribution of progeny from intercrosses between $Gata2^{f/+}; Tg^{OVE-Cre}$ and $Gata2^{f/+}$.

Genotype	f/f		f/+		+/+	
	Tg ⁻	Tg ⁺	Tg ⁻	Tg ⁺	Tg ⁻	Tg ⁺
4 week	12	11	18	21	10	9

f/f, $Gata2^{f/f}$; f/+, $Gata2^{f/+}$; +/+, $Gata2^{+/+}$; Tg, $Tg^{OVE-Cre}$

Table 2. Sequence of primers used in RT-qPCR

Gene	Sense primer	Antisense primer	Assay
<i>TrkC</i>	CTGAGTGCTACAATCTAAGCCC	CACACCCCATAGAACTTGACAAT	RT-qPCR
<i>Bdnf</i>	TGCAGGGGCATAGACAAAAGG	CTTATGAATCGCCAGCCAATTCTC	RT-qPCR
<i>NT3</i>	GGAGTTTGCCGGAAGACTCTC	GGGTGCTCTGGTAATTTTCCTTA	RT-qPCR
<i>NeuroD</i>	ACTACCTGTGACCTTTCCCA	GAAGTGCTAAGGCAACGCAA	RT-qPCR
<i>TrkB</i>	AAGGACTTTCATCGGGAAGCTG	TCGCCCTCCACACAGACAC	RT-qPCR
<i>Gata2</i>	ACCTGTGCAATGCCTGTGGG	TTGCACAACAGGTGCCCGCT	RT-qPCR
<i>Gata3</i>	GGTGGACGTACTTTTAAACATCGA	CCCTGACGGAGTTTCCGTAG	RT-qPCR
<i>rRNA</i>	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT	RT-qPCR

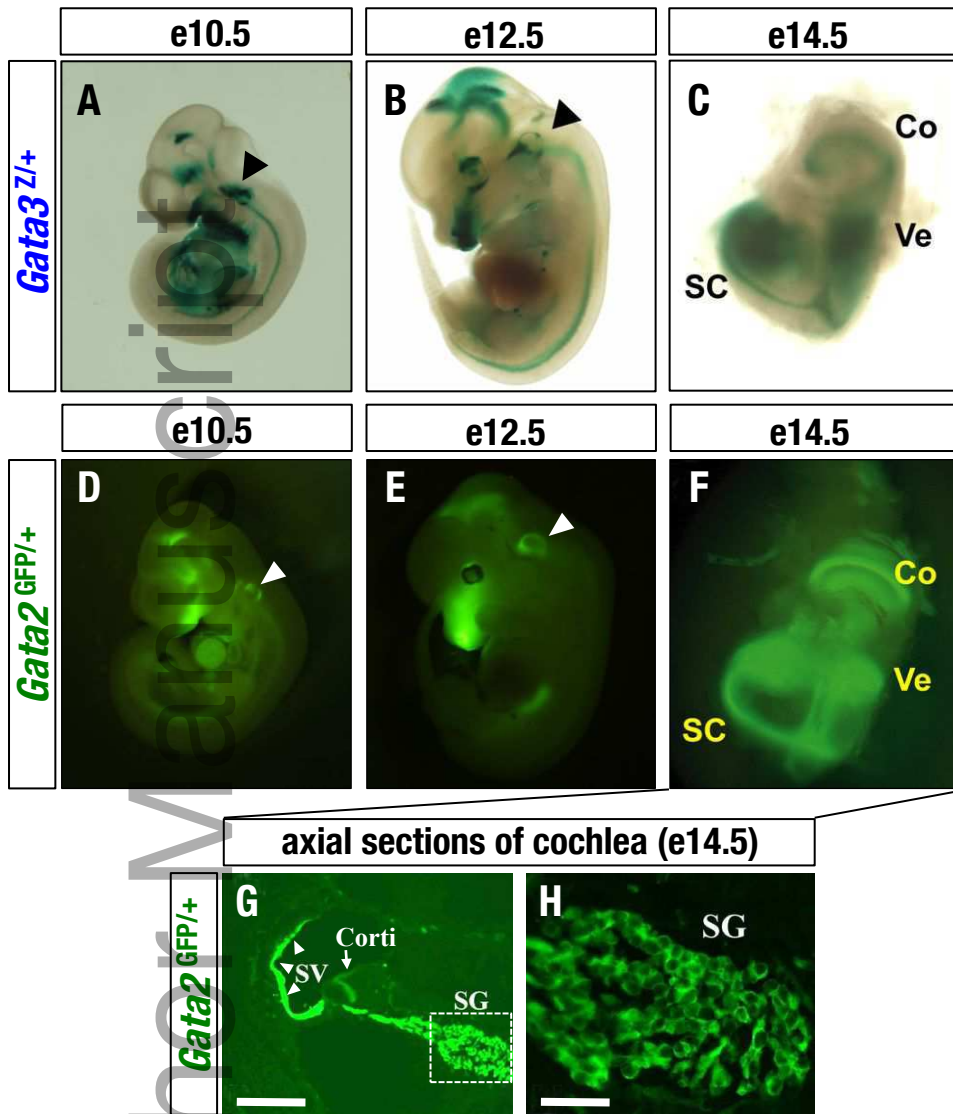


Fig. 1. Expression of GATA2 and GATA3 during the inner ear development.

(A-B) *Gata3^{Z/+}* embryos showed *LacZ* expression in the otic vesicle (arrowheads) at e10.5 and e12.5dpc. (C) *LacZ* expression was detected in the cochlea (Co), vestibules (Ve), and semicircular canals (SC) in the dissected inner ear tissue of e14.5 *Gata3^{Z/+}* embryo. (D-F) GFP-fluorescence was detected in the otic vesicle (arrowhead) of *Gata2^{GFP/+}* embryos at e10.5 and e12.5. Inner ear tissue of the e14.5 *Gata2^{GFP/+}* embryos showed GFP fluorescence in the cochlea, vestibule, and semicircular canals. (G) An axial section of the cochlea of an e14.5 *Gata2^{GFP/+}* embryo showed robust GFP expression in the spiral ganglion (SG, dotted rectangle) and stria vascularis (SV, arrowheads), while organ of Corti (arrow) showed only weak GFP fluorescence. (H) Higher magnification of the spiral ganglion showed a collection of GFP-positive cells. Scale bars; 40 μ m (G), 10 μ m (H).

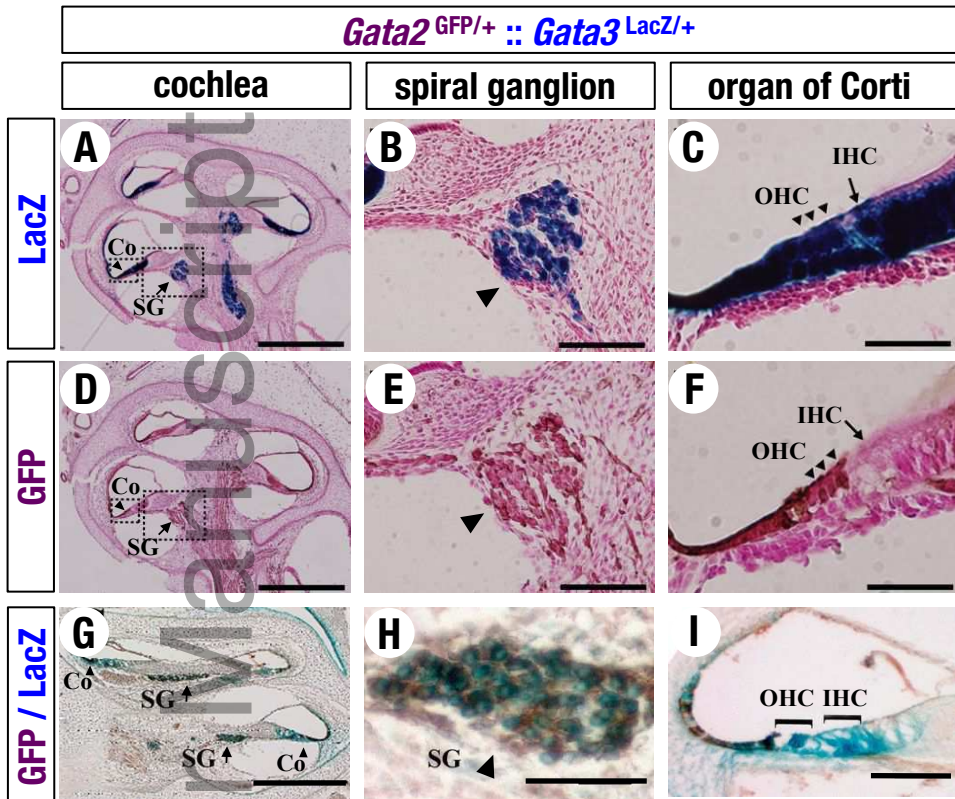


Fig. 2. GATA2 and GATA3 are co-expressed in the spiral ganglion cells in the cochlea. In the cochlea of postnatal day 3 (P3) *Gata2*^{GFP/+}::*Gata3*^{LacZ/+} mice, GFP-immunoreactivity (brown) and Xgal-staining signals (blue) were detected in the spiral ganglion (SG) and organ of Corti (Co) (A, D and G). At higher magnification, the GFP- and *LacZ*-expression was colocalized in the vast majority of the spiral ganglion cells (arrowheads in B, E and H). (C, F and I) In the organ of Corti, robust *LacZ*-expression was detected in the both outer hair cells (OHC) and inner hair cells (IHC) (C), while modest GFP-expression was detected, but only in the OHC. Scale bars: (A) and (D) ; 500 μ m. (B) and (E) ; 200 μ m. (C) and (F); 100 μ m. (G); 200 μ m. (H); 20 μ m. (I); 40 μ m.

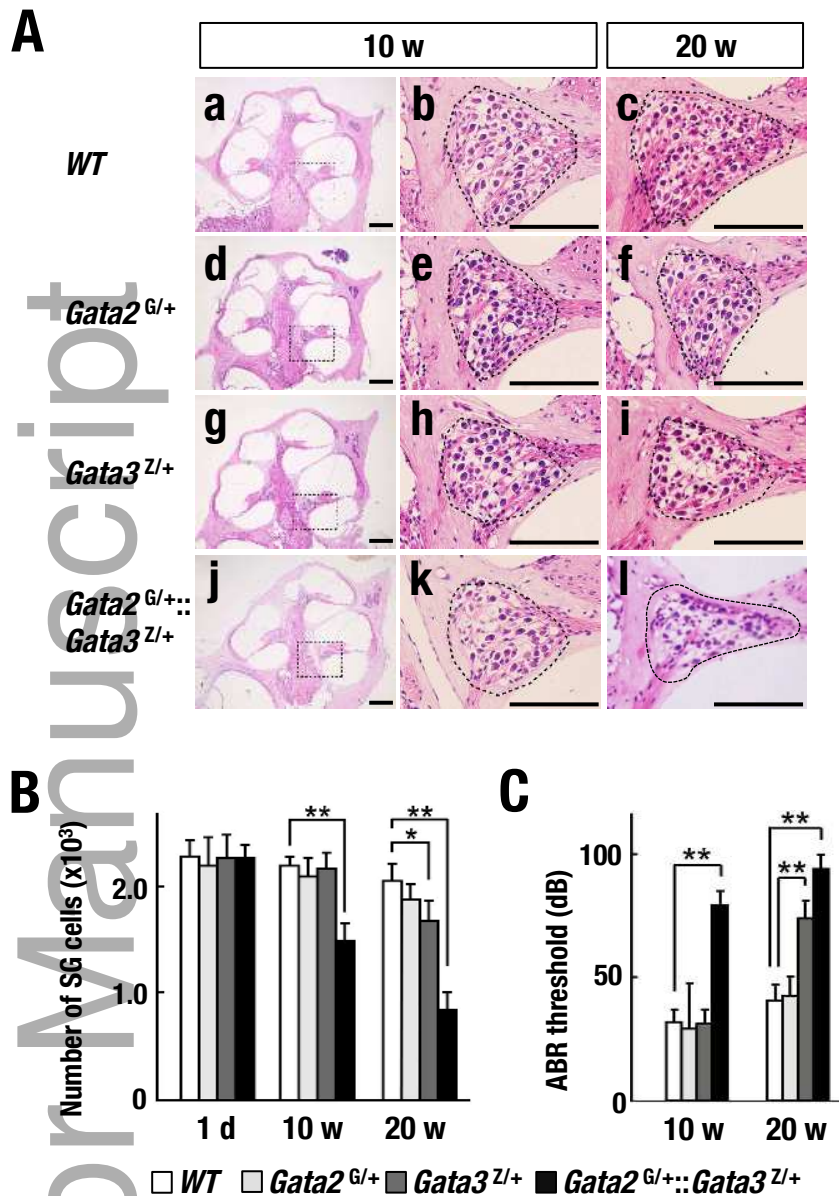


Fig. 3 *Gata2^{G/+::Gata3^{Z/+}}* compound heterozygous mutant mice show progressive spiral ganglion cell loss and hearing impairment with age. (A) Axial sections of the cochlea in each genotype of mice show comparable gross architecture of the cochlear canal at 10 weeks of age (a, d, g and j). *Gata2^{G/+::Gata3^{Z/+}}* mice showed a progressively diminished number of spiral ganglion cells at 10 and 20 weeks of age (k and l) in comparison with the age-matched wild-type control (b and c). *Gata3^{Z/+}* heterozygous mutant mice had a modest reduction in the spiral ganglion cell population at 20 weeks of age (i). *Gata2^{G/+}* (10 weeks and 20 weeks) and *Gata3^{Z/+}* (10 weeks) mice appeared to have normal cellularity in the spiral ganglion (e, f and h). Scale bars: 200 μ m. (B) Quantitative analyses of the number of residual spiral ganglion cells per cochlea at postnatal day 1 or after 10 weeks or 20 weeks of age. *Gata2^{G/+::Gata3^{Z/+}}* mice had a progressive decrease of the spiral ganglion cell population at 10 and 20 weeks of age. *Gata3^{Z/+}* heterozygous mutant mice showed a modest decrease in spiral ganglion cell number at 20 weeks of age. (C) Click-evoked ABR thresholds of each genotype of mice at 10 and 20 weeks of age. ABR thresholds of *Gata2^{G/+::Gata3^{Z/+}}* mice at 10 weeks of age were significantly more elevated than those in the age-matched wild-type mice. The age-matched *Gata2^{G/+}* or *Gata3^{Z/+}* heterozygous mutants showed normal ABR threshold at 10 weeks of age. ABR thresholds of *Gata2^{G/+::Gata3^{Z/+}}* mice was even more elevated in the 20 week old animals. *Gata3^{Z/+}* heterozygous mutant mice also showed increased ABR thresholds at 20 weeks of age. Data are presented as mean \pm SD. The statistical significance of differences are indicated (* $P < 0.05$, ** $P < 0.01$; Student's *t*-test). More than five mice in each group were subjected to statistical analysis.

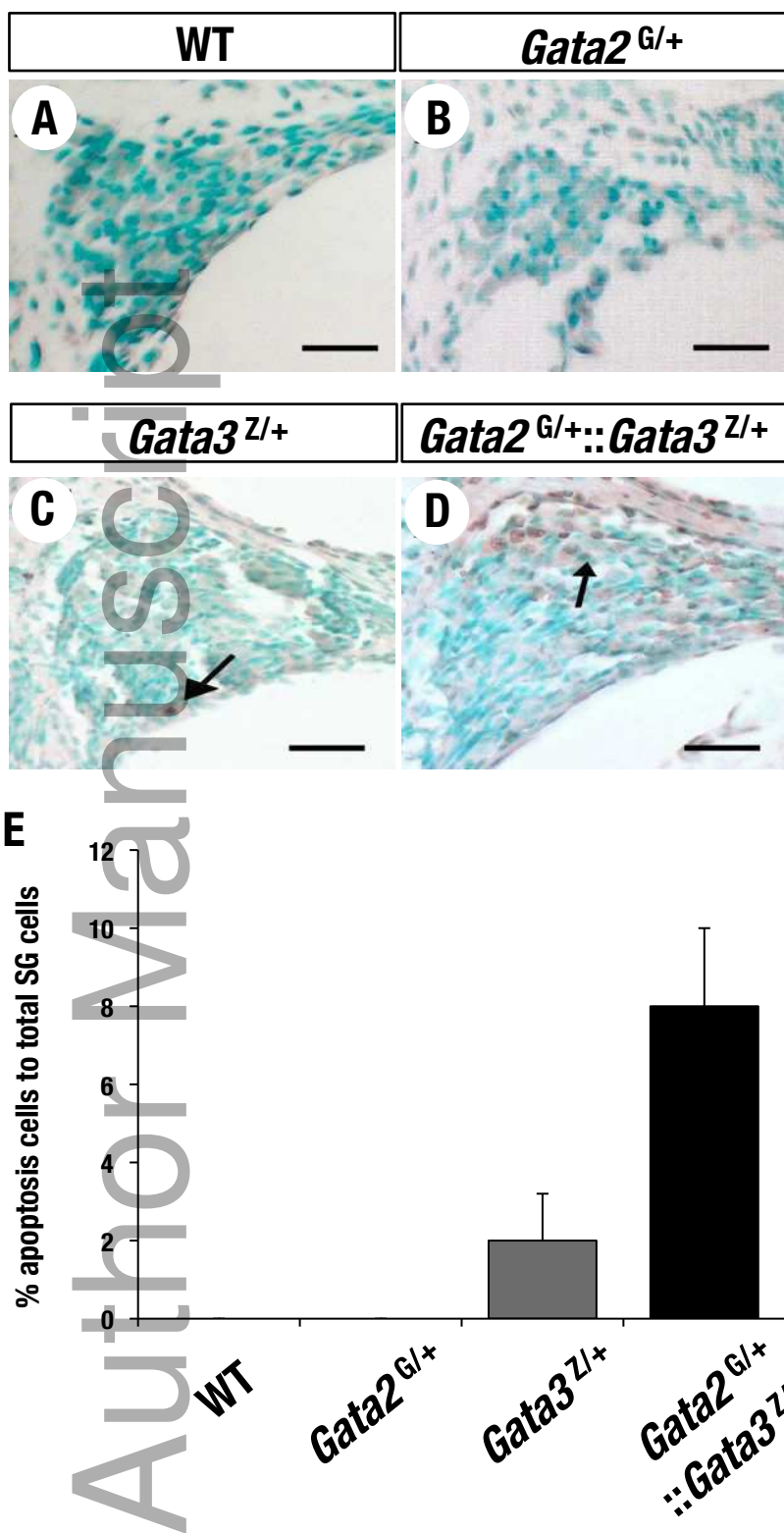


Fig. 4. Enhanced apoptosis in the spiral ganglion of *Gata2*^{G/+}::*Gata3*^{Z/+} mice. A number of TUNEL-positive cells were found in the spiral ganglion of *Gata2*^{G/+}::*Gata3*^{Z/+} mice (D). In contrast, no TUNEL-positive cells were detected in the spiral ganglia of wild-type or *Gata2*^{G/+} mice (A and B). *Gata3*^{Z/+} had a modest increase in apoptotic cells in the spiral ganglion (C). Scale bars = 10 μ m. (E) Percentage of TUNEL-positive cells among the total spiral ganglion cells enumerated in five sections of each genotype of mice. Data represent mean \pm SD.

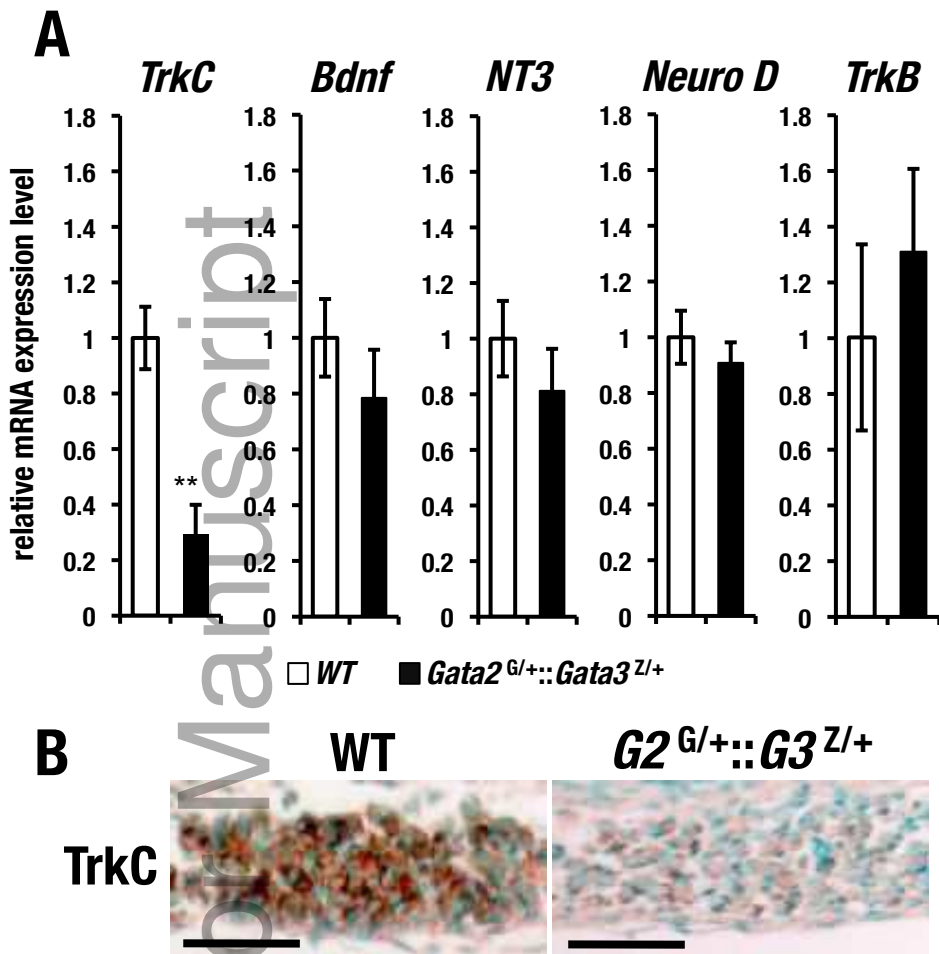


Fig. 5. mRNA expression of neurotrophin signaling-related genes. (A) *TrkC* expression is markedly reduced in the *Gata2*^{G/+}::*Gata3*^{Z/+} compound mutant mice compared to wild-type control mice. *TrkB* tended to be higher in the *Gata2*^{G/+}::*Gata3*^{Z/+} mice, albeit below statistical significance. *Bdnf* and *NT3* levels were not significantly changed, and *NeuroD* was also unaffected. The statistical significance of differences are indicated (** $P < 0.01$; Student's *t*-test). (B) *TrkC* immunoreactivity was markedly reduced in the spiral ganglia of the *Gata2*^{G/+}::*Gata3*^{Z/+} mice (right), whereas wild-type mice abundantly expressed *TrkC* (left). Scale bars = 20 μ m

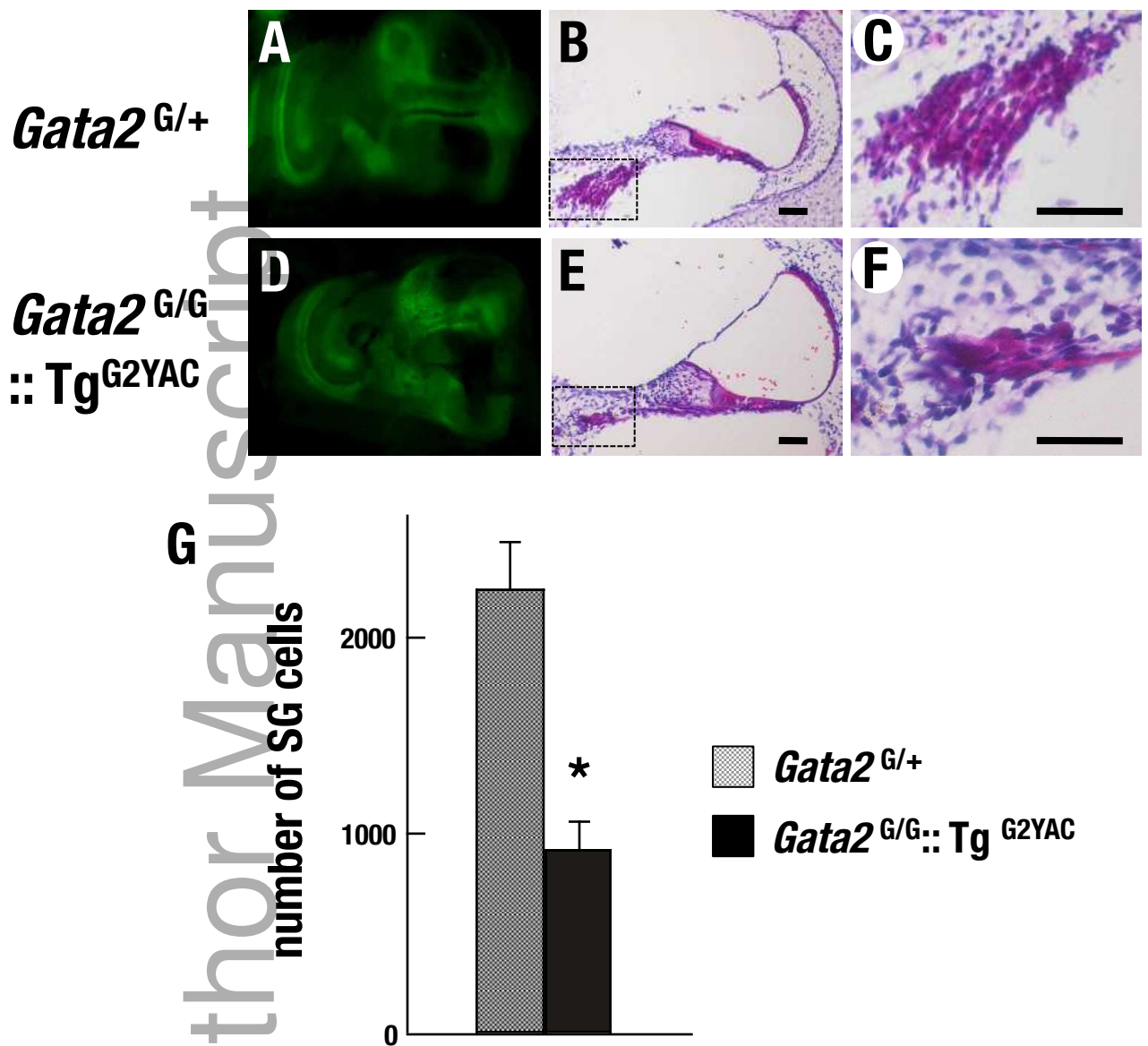


Fig. 6. Tg^{G2YAC}-rescued *Gata2* null-deficient neonates showed diminished numbers of spiral ganglion cells. No gross abnormality was found in the GFP-expression pattern in the cochlea of *Gata2*^{G/G} :: Tg^{G2YAC} mice in comparison with the *Gata2*^{G/+} cochlea (compare A and D). *Gata2*^{G/G} :: Tg^{G2YAC} mice showed a significant reduction of spiral ganglion cells in comparison with the *Gata2*^{G/+} control mice by HE-staining comparison (B, C E and F). Scale bars represent 10 μm. (G) Number of the spiral ganglion cells in the *Gata2*^{G/G} :: Tg^{G2YAC} mice was statistically significantly lower than that of the *Gata2*^{G/+} control mice. The statistical significance of differences are indicated (* *P* < 0.05; Student's *t* test). More than five mice of each genotype were subjected to the analysis.

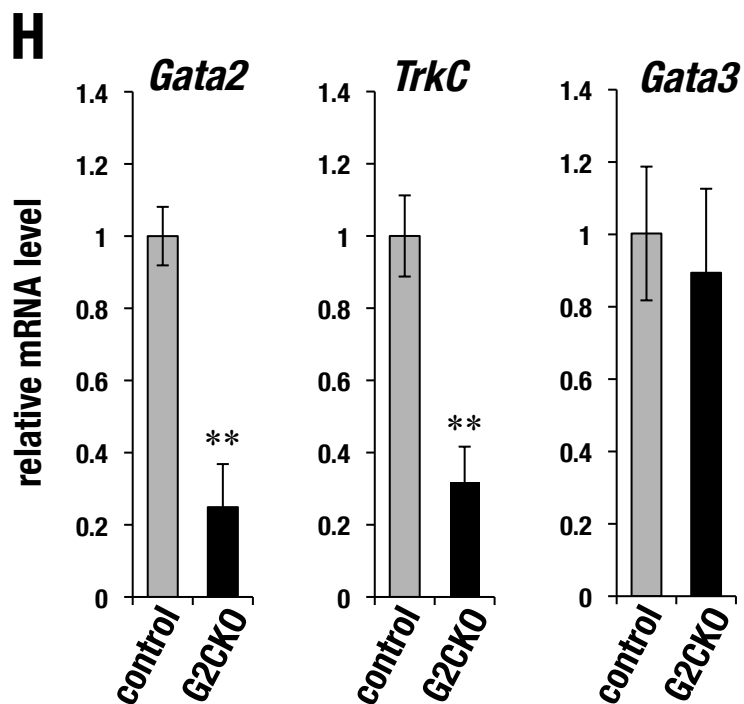
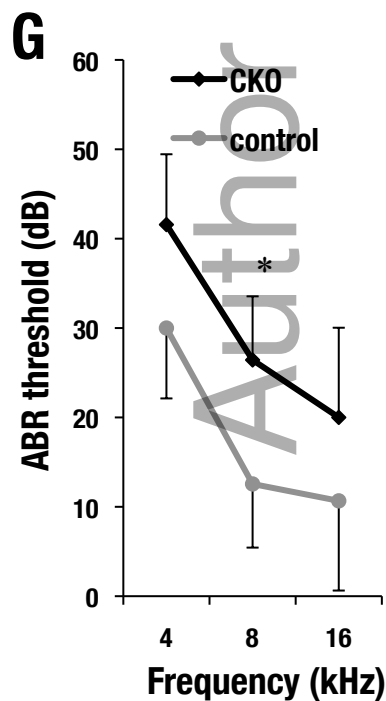
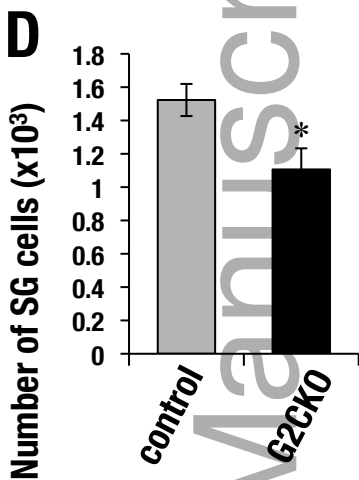
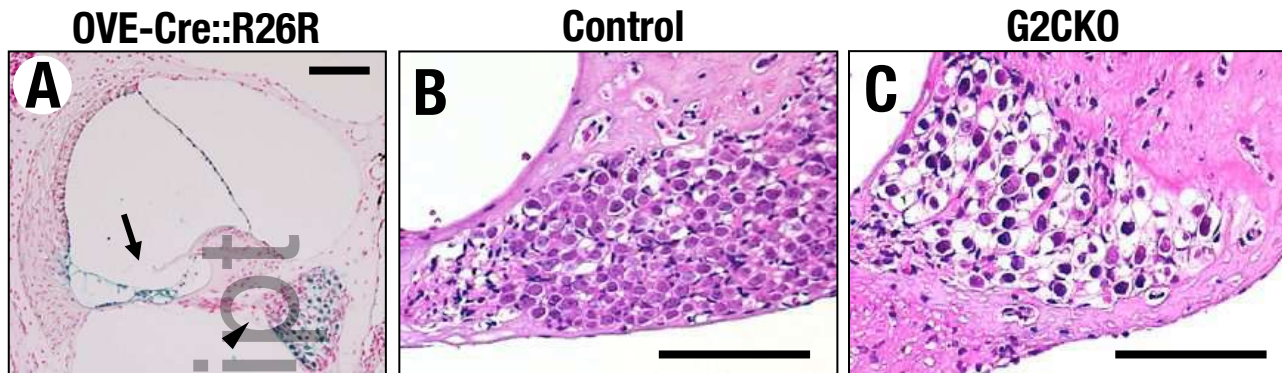


Fig. 7. Hearing loss in the inner ear-specific *Gata2*-deficient (G2CKO) mice. (A) $Tg^{OVE-Cre}$ mice crossed with ROSA26 floxed *LacZ* (R26R) reporter mice showed *lacZ* expression in the spiral ganglion cells (arrowhead) and organ of Corti (arrow). (B and C) $Gata2^{f/f};Tg^{OVE-Cre}$ (G2CKO) mice showed diminished spiral ganglion cell numbers at 20 weeks of age in comparison to age matched wild-type control mice. (D) Quantification of the spiral ganglion cell number confirmed a statistically significant decrease in the G2CKO mice (n=10) compared with controls (n=14). (E and F) The hair cell population in the organ of Corti in the G2CKO mice was comparable to that in the control. (G) Pure tone bursts (4, 8 or 16 kHz)-evoked ABR thresholds in mutant (CKO) or wild type (control) mice at 20 weeks of age. The ABR thresholds of G2CKO mice were significantly more elevated than those in the age-matched wild-type mice. (H) *Gata2* mRNA is reduced to 24.9% in the G2CKO mice compared to that in the wild-type control mice. Similarly, *TrkC* expression is reduced in the G2CKO mice. *GATA3* mRNA expression level is not significantly different between the genotypes. The statistical significance of differences are indicated (* $P < 0.05$, ** $P < 0.01$; Student's *t*-test). The data represent the mean \pm SD (n = 6-8). Scale bars: (A)-(C), 200 μ m; (E)-(F), 50 μ m.