Grosh, K., Zheng, J., Zou, Y., de Boer, E., & Nuttall, A. L. (2004). High-frequency electromotile responses in the cochlea. *The Journal of the Acoustical Society of America*, 115(5 Pt 1), 2178–2184.

Gunther, T., Ising, H., & Joachims, Z. (1989). Biochemical mechanisms affecting susceptibility to noise-induced hearing loss. *The American Journal of Otology*, 10(1), 36–41.

Halliwell, B., & Gutteridge, J. M. (1986). Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts. *Archives of Biochemistry & Biophysics*, 246(2), 501–514.

Halliwell, B., & Gutteridge, J. M. C. (2007). *Free radicals in biology and medicine* (4th ed.). New York: Oxford University Press.

Hansen, M. R., Zha, X. M., Bok, J., & Green, S. H. (2001). Multiple distinct signal pathways, including an autocrine neurotrophic mechanism, contribute to the survival-promoting effect of depolarization on spiral ganglion neurons in vitro. *The Journal of Neuroscience*, 21(7), 2256–2267.

Hansen, M. R., Bok, J., Devaiah, A. K., Zha, X. M., & Green, S. H. (2003). Ca^{2+}/calmodulin-dependent protein kinases II and IV both promote survival but differ in their effects on axon growth in spiral ganglion neurons. *Journal of Neuroscience Research*, 72(2), 169–184.

Hayashida, K., Sano, M., Ohsawa, I., Shinmura, K., Tamaki, K., Kimura, K., & Fukuda, K. (2008). Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. *Biochemical and Biophysical Research Communications*, 373(1), 30–35.

Henderson, D., Bielefeld, E. C., Harris, K. C., & Hu, B. H. (2006). The role of oxidative stress in noise-induced hearing loss. *Ear & Hearing*, 27(1), 1–19.

Hirose, Y., Sugahara, K., Mikuriya, T., Hashimoto, M., Shimogori, H., & Yamashita, H. (2008). Effect of water-soluble coenzyme Q10 on noise-induced hearing loss in guinea pigs. *Acta Oto-Laryngologica*, 128(10), 1071–1076.

Hossain, W. A., & Morest, D. K. (2000). Fibroblast growth factors (FGF-1, FGF-2) promote migration and neurite growth of mouse cochlear ganglion cells in vitro: Immunohistochemistry and antibody perturbation. *Journal of Neuroscience Research*, 62(1), 40–55.

Hou, F., Wang, S., Zhai, S., Hu, Y., Yang, W., & He, L. (2003). Effects of alpha-tocopherol on noise-induced hearing loss in guinea pigs. *Hearing Research*, 179(1–2), 1–8.

Hu, B. H., Zheng, X. Y., McFadden, S. L., Kopke, R. D., & Henderson, D. (1997). R-phenylisopropyladenosine attenuates noise-induced hearing loss in the chinchilla. *Hearing Research*, 113(1–2), 198–206.

Hu, Z., Ulfendahl, M., & Olivius, N. P. (2004a). Central migration of neuronal tissue and embryonic stem cells following transplantation along the adult auditory nerve. *Brain Research*, 1026(1), 68–73.

Hu, Z., Ulfendahl, M., & Olivius, N. P. (2004b). Survival of neuronal tissue following xenograft implantation into the adult rat inner ear. *Experimental Neurology*, 185(1), 7–14.

Hu, Z., Andang, M., Ni, D., & Ulfendahl, M. (2005a). Neural cograft stimulates the survival and differentiation of embryonic stem cells in the adult mammalian auditory system. *Brain Research*, 1051(1–2), 137–144.

Hu, Z., Wei, D., Johansson, C. B., Holmstrom, N., Duan, M., Frisen, J., & Ulfendahl, M. (2005b). Survival and neural differentiation of adult neural stem cells transplanted into the mature inner ear. *Experimental Cell Research*, 302(1), 40–47.

Hu, Z., Ulfendahl, M., Prieskorn, D. M., Olivius, P., & Miller, J. M. (2009). Functional evaluation of a cell replacement therapy in the inner ear. *Otology & Neurotology*, 30(4), 551–558.

Huang, C., Li, J., Costa, M., Zhang, Z., Leonard, S. S., Castranova, V., & Shi, X. (2001). Hydrogen peroxide mediates activation of nuclear factor of activated T cells (NFAT) by nickel subsulfide. *Cancer Research*, 61(22), 8051–8057.

Husseman, J., & Raphael, Y. (2009). Gene therapy in the inner ear using adenovirus vectors. *Advances in Oto-Rhino-Laryngology*, 66, 37–51.

Iguchi, F., Nakagawa, T., Tateya, I., Endo, T., Kim, T. S., Dong, Y., & Ito, J. (2004). Surgical techniques for cell transplantation into the mouse cochlea. *Acta Oto-Laryngologica*, (551, Supplement), 43–47.

Incesulu, A., & Nadol, J. B., Jr. (1998). Correlation of acoustic threshold measures and spiral ganglion cell survival in severe to profound sensorineural hearing loss: Implications for cochlear implantation. *The Annals of Otology, Rhinology, & Laryngology*, 107(11 Pt 1), 906–911.

Ito, J., Murata, M., & Kawaguchi, S. (2001). Regeneration and recovery of the hearing function of the central auditory pathway by transplants of embryonic brain tissue in adult rats. *Experimental Neurology*, 169(1), 30–35.

Izumikawa, M., Minoda, R., Kawamoto, K., Abrashkin, K. A., Swiderski, D. L., Dolan, D. F., & . Raphael, Y. (2005). Auditory hair cell replacement and hearing improvement by *Atoh1* gene therapy in deaf mammals. *Nature Medicine*, 11(3), 271–276.

Kajiyama, S., Hasegawa, G., Asano, M., Hosoda, H., Fukui, M., Nakamura, N., & Yoshikawa, T. (2008). Supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance. *Nutrition Research*, 28(3), 137–143.

Kanzaki, S., Stover, T., Kawamoto, K., Prieskorn, D. M., Altschuler, R. A., Miller, J. M., & Raphael, Y. (2002). Glial cell line-derived neurotrophic factor and chronic electrical stimulation prevent VIII cranial nerve degeneration following denervation. *The Journal of Comparative Neurology*, 454(3), 350–360.

Kashio, A., Sakamoto, T., Suzukawa, K., Asoh, S., Ohta, S., & Yamasoba, T. (2007). A protein derived from the fusion of TAT peptide and FNK, a Bcl-x(L) derivative, prevents cochlear hair cell death from aminoglycoside ototoxicity in vivo. *Journal of Neuroscience Research*, 85(7), 1403–1412.

Kawamoto, K., Kanzaki, S., Yagi, M., Stover, T., Prieskorn, D. M., Dolan, D. F., & Raphael, Y. (2001). Gene-based therapy for inner ear disease. *Noise Health*, 3(11), 37–47.

Kawamoto, K., Yagi, M., Stover, T., Kanzaki, S., & Raphael, Y. (2003). Hearing and hair cells are protected by adenoviral gene therapy with TGF-beta1 and GDNF. *Molecular Therapy*, 7(4), 484–492.

Kawamoto, K., Sha, S. H., Minoda, R., Izumikawa, M., Kuriyama, H., Schacht, J., & Raphael, Y. (2004). Antioxidant gene therapy can protect hearing and hair cells from ototoxicity. *Molecular Therapy*, 9(2), 173–181.

Kikkawa, Y. S., Nakagawa, T., Horie, R. T., & Ito, J. (2009). Hydrogen protects auditory hair cells from free radicals. *NeuroReport*, 20(7), 689–694.

Kirkland, R. A., & Franklin, J. L. (2003). Bax, reactive oxygen, and cytochrome *c* release in neuronal apoptosis. *Antioxidants & Redox Signaling*, 5(5), 589–596.

Kopke, R. D., Weisskopf, P. A., Boone, J. L., Jackson, R. L., Wester, D. C., Hoffer, M. E., & McBride, D. (2000). Reduction of noise-induced hearing loss using L-NAC and salicylate in the chinchilla. *Hearing Research*, 149(1–2), 138–146.

Kopke, R. D., Coleman, J. K., Liu, J., Campbell, K. C., & Riffenburgh, R. H. (2002). Candidate's thesis: Enhancing intrinsic cochlear stress defenses to reduce noise-induced hearing loss. *The Laryngoscope*, 112(9), 1515–1532.

Küçük, B., Abe, K., Ushiki, T., Inuyama, Y., Fukuda, S., & Ishikawa, K. (1991). Microstructures of the bony modiolus in the human cochlea: A scanning electron microscopic study. *Journal of Electron Microscopy (Tokyo)*, 40(3), 193–197.

Kujawa, S. G., & Liberman, M. C. (2009). Adding insult to injury: Cochlear nerve degeneration after "temporary" noise-induced hearing loss. *The Journal of Neuroscience*, 29(45), 14077–14085.

Kwan, T., White, P. M., & Segil, N. (2009). Development and regeneration of the inner ear. *Annals of the New York Academy of Sciences*, 1170, 28–33.

Lamm, K., & Arnold, W. (2000). The effect of blood flow promoting drugs on cochlear blood flow, perilymphatic pO_2 and auditory function in the normal and noise-damaged hypoxic and ischemic guinea pig inner ear. *Hearing Research*, 141(1–2), 199–219.

Lawner, B. E., Harding, G. W., & Bohne, B. A. (1997). Time course of nerve-fiber regeneration in the noise-damaged mammalian cochlea. *International Journal of Developmental Neuroscience*, 15(4–5), 601–617.

Le Prell, C. G., Yagi, M., Kawamoto, K., Beyer, L. A., Atkin, G., Raphael, Y., & Moody, D. B. (2004). Chronic excitotoxicity in the guinea pig cochlea induces temporary functional deficits without disrupting otoacoustic emissions. *The Journal of the Acoustical Society of America*, 116(2), 1044–1056.

Le Prell, C. G., Hughes, L. F., & Miller, J. M. (2007a). Free radical scavengers vitamins A, C, and E plus magnesium reduce noise trauma. *Free Radical Biology & Medicine*, 42(9), 1454–1463.

Le Prell, C. G., Yamashita, D., Minami, S. B., Yamasoba, T., & Miller, J. M. (2007b). Mechanisms of noise-induced hearing loss indicate multiple methods of prevention. *Hearing Research*, 226(1–2), 22–43.

Lenarz, T. (2009). Electro-acoustic stimulation of the cochlea. Editorial. *Audiology & Neuro-otology*, 14 (Supplement 1), 1.

Levine, M., Conry-Cantilena, C., Wang, Y., Welch, R. W., Washko, P. W., Dhariwal, K. R., & Cantilena, L. R. (1996). Vitamin C pharmacokinetics in healthy volunteers: Evidence for a recommended dietary allowance. *Proceedings of the National Academy of Sciences of the USA*, 93(8), 3704–3709.

Li, H., Liu, H., & Heller, S. (2003a). Pluripotent stem cells from the adult mouse inner ear. *Nature Medicine*, 9(10), 1293–1299.

Li, H., Roblin, G., Liu, H., & Heller, S. (2003b). Generation of hair cells by stepwise differentiation of embryonic stem cells. *Proceedings of the National Academy of Sciences of the USA*, 100(23), 13495–13500.

Li, H., Corrales, C. E., Edge, A., & Heller, S. (2004). Stem cells as therapy for hearing loss. *Trends in Molecular Medicine*, 10(7), 309–315.

Lim, H. H., Lenarz, T., Anderson, D. J., & Lenarz, M. (2008). The auditory midbrain implant: Effects of electrode location. *Hearing Research*, 242(1–2), 74–85.

Lim, H. H., Lenarz, M., & Lenarz, T. (2009). Auditory midbrain implant: A review. *Trends in Amplification*, 13(3), 149–180.

Lopez, I. A., Zhao, P. M., Yamaguchi, M., de Vellis, J., & Espinosa-Jeffrey, A. (2004). Stem/progenitor cells in the postnatal inner ear of the GFP-nestin transgenic mouse. *International Journal of Developmental Neuroscience*, 22(4), 205–213.

Lynch, E. D., & Kil, J. (2005). Compounds for the prevention and treatment of noise-induced hearing loss. *Drug Discovery Today*, 10(19), 1291–1298.

Maricich, S. M., Xia, A., Mathes, E. L., Wang, V. Y., Oghalai, J. S., Fritzsch, B., & Zoghbi, H. Y. (2009). *Atoh1*-lineal neurons are required for hearing and for the survival of neurons in the spiral ganglion and brainstem accessory auditory nuclei. *The Journal of Neuroscience*, 29(36), 11123–11133.

Martinez-Monedero, R., Yi, E., Oshima, K., Glowatzki, E., & Edge, A. S. (2008). Differentiation of inner ear stem cells to functional sensory neurons. *Developmental Neurobiology*, 68(5), 669–684.

Maruyama, J., Yamagata, T., Ulfendahl, M., Bredberg, G., Altschuler, R. A., & Miller, J. M. (2007). Effects of antioxidants on auditory nerve function and survival in deafened guinea pigs. *Neurobiology of Disease*, 25(2), 309–318.

Maruyama, J., Miller, J. M., & Ulfendahl, M. (2008). Glial cell line-derived neurotrophic factor and antioxidants preserve the electrical responsiveness of the spiral ganglion neurons after experimentally induced deafness. *Neurobiology of Disease*, 29(1), 14–21.

Mattson, M. P. (1998). Neuroprotective strategies based on targeting of postreceptor signaling events. In M. P. Mattson (Ed.), *Neuroprotective signal transduction* (pp. 301–335). Totowa, NJ: Humana Press.

McFadden, S. L., Ding, D., Jiang, H., & Salvi, R. J. (2004). Time course of efferent fiber and spiral ganglion cell degeneration following complete hair cell loss in the chinchilla. *Brain Research*, 997(1), 40–51.

McFadden, S. L., Woo, J. M., Michalak, N., & Ding, D. (2005). Dietary vitamin C supplementation reduces noise-induced hearing loss in guinea pigs. *Hearing Research*, 202(1–2), 200–208.

Middlebrooks, J. C., & Snyder, R. L. (2007). Auditory prosthesis with a penetrating nerve array. *Journal of the Association for Research in Otolaryngology*, 8(2), 258–279.

Miller, J. M., Chi, D. H., O'Keeffe, L. J., Kruszka, P., Raphael, Y., & Altschuler, R. A. (1997). Neurotrophins can enhance spiral ganglion cell survival after inner hair cell loss. *International Journal of Developmental Neuroscience*, 15(4–5), 631–643.

Miller, J. M., Miller, A. L., Yamagata, T., Bredberg, G., & Altschuler, R. A. (2002). Protection and regrowth of the auditory nerve after deafness: Neurotrophins, antioxidants and depolarization are effective in vivo. *Audiology & Neuro-otology*, 7(3), 175–179.

Miller, A. L., Prieskorn, D. M., Altschuler, R. A., & Miller, J. M. (2003a). Mechanism of electrical stimulation-induced neuroprotection: Effects of verapamil on protection of primary auditory afferents. *Brain Research*, 966(2), 218–230.

Miller, J. M., Brown, J. N., & Schacht, J. (2003b). 8-iso-prostaglandin $F_2\alpha$, a product of noise exposure, reduces inner ear blood flow. *Audiology & Neuro-otology*, 8(4), 207–221.

Miller, J., Yamashita, D., Minami, S., Yamasoba, T., & Le Prell, C. (2006). Mechanisms and prevention of noise-induced hearing loss. *Otology Japan*, 16(2), 139–153.

Miller, J. M., Le Prell, C. G., Prieskorn, D. M., Wys, N. L., & Altschuler, R. A. (2007). Delayed neurotrophin treatment following deafness rescues spiral ganglion cells from death and promotes regrowth of auditory nerve peripheral processes: Effects of brain-derived neurotrophic factor and fibroblast growth factor. *Journal of Neuroscience Research*, 85(9), 1959–1969.

Minami, S. B., Yamashita, D., Ogawa, K., Schacht, J., & Miller, J. M. (2007). Creatine and tempol attenuate noise-induced hearing loss. *Brain Research*, 1148, 83–89.

Morris, M. C., Depollier, J., Mery, J., Heitz, F., & Divita, G. (2001). A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nature Biotechnology*, 19(12), 1173–1176.

Munson, B., & Nelson, P. B. (2005). Phonetic identification in quiet and in noise by listeners with cochlear implants. *The Journal of the Acoustical Society of America*, 118(4), 2607–2617.

Nadol, J. B., Jr., Young, Y. S., & Glynn, R. J. (1989). Survival of spiral ganglion cells in profound sensorineural hearing loss: Implications for cochlear implantation. *The Annals of Otology, Rhinology & Laryngology*, 98(6), 411–416.

Nagata, K., Nakashima-Kamimura, N., Mikami, T., Ohsawa, I., & Ohta, S. (2009). Consumption of molecular hydrogen prevents the stress-induced impairments in hippocampus-dependent learning tasks during chronic physical restraint in mice. *Neuropsychopharmacology*, 34(2), 501–508.

Naito, Y., Nakamura, T., Nakagawa, T., Iguchi, F., Endo, T., Fujino, K., & Ito, J. (2004). Transplantation of bone marrow stromal cells into the cochlea of chinchillas. *NeuroReport*, 15(1), 1–4.

Neef, D. W., Turski, M. L., & Thiele, D. J. (2010). Modulation of heat shock transcription factor 1 as a therapeutic target for small molecule intervention in neurodegenerative disease. *PLoS Biology*, 8(1), e1000291.

Nishimura, K., Nakagawa, T., Ono, K., Ogita, H., Sakamoto, T., Yamamoto, N., & Ito, J. (2009). Transplantation of mouse induced pluripotent stem cells into the cochlea. *NeuroReport*, 20(14), 1250–1254.

Ohinata, Y., Yamasoba, T., Schacht, J., & Miller, J. M. (2000). Glutathione limits noise-induced hearing loss. *Hearing Research*, 146(1–2), 28–34.

Ohinata, Y., Miller, J. M., & Schacht, J. (2003). Protection from noise-induced lipid peroxidation and hair cell loss in the cochlea. *Brain Research*, 966(2), 265–273.

Ohlemiller, K. K., Wright, J. S., & Dugan, L. L. (1999). Early elevation of cochlear reactive oxygen species following noise exposure. *Audiology & Neuro-otology*, 4(5), 229–236.

Ohsawa, I., Ishikawa, M., Takahashi, K., Watanabe, M., Nishimaki, K., Yamagata, K., & Ohta, S. (2007). Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nature Medicine*, 13(6), 688–694.

Oliva, E. N., Ronco, F., Marino, A., Alati, C., Pratico, G., & Nobile, F. (2010). Iron chelation therapy associated with improvement of hematopoiesis in transfusion-dependent patients. *Transfusion*, 50(7), 1568–1570.

Oshima, K., Shin, K., Diensthuber, M., Peng, A. W., Ricci, A. J., & Heller, S. (2010). Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells. *Cell*, 141(4), 704–716.

Patsch, C., & Edenhofer, F. (2007). Conditional mutagenesis by cell-permeable proteins: Potential, limitations and prospects. Handbook of Experimental. *Pharmacology*, (178), 203–232.

Pirvola, U., Hallbook, F., Xing-Qun, L., Virkkala, J., Saarma, M., & Ylikoski, J. (1997). Expression of neurotrophins and Trk receptors in the developing, adult, and regenerating avian cochlea. *Journal of Neurobiology*, 33(7), 1019–1033.

Pourbakht, A., & Yamasoba, T. (2003). Ebselen attenuates cochlear damage caused by acoustic trauma. *Hearing Research*, 181(1–2), 100–108.

Probst, F. J., Fridell, R. A., Raphael, Y., Saunders, T. L., Wang, A., Liang, Y., & Camper, S. A. (1998). Correction of deafness in shaker-2 mice by an unconventional myosin in a BAC transgene. *Science*, 280(5368), 1444–1447.

Puel, J. L., Pujol, R., Ladrech, S., & Eybalin, M. (1991). Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid electrophysiological and neurotoxic effects in the guinea-pig cochlea. *Neuroscience*, 45(1), 63–72.

Puel, J. L., Saffiedine, S., Gervais d'Aldin, C., Eybalin, M., & Pujol, R. (1995). Synaptic regeneration and functional recovery after excitotoxic injury in the guinea pig cochlea. *Comptes Rendus de l' Académie des Sciences Série III*, 318(1), 67–75.

Puel, J. L., Ruel, J., Gervais d'Aldin, C., & Pujol, R. (1998). Excitotoxicity and repair of cochlear synapses after noise-trauma induced hearing loss. *NeuroReport*, 9(9), 2109–2114.

Pujol, R., & Puel, J. L. (1999). Excitotoxicity, synaptic repair, and functional recovery in the mammalian cochlea: A review of recent findings. *Annals of the New York Academy of Sciences*, 884, 249–254.

Quirk, W. S., & Seidman, M. D. (1995). Cochlear vascular changes in response to loud noise. *The American Journal of Otology*, 16(3), 322–325.

Quirk, W. S., Avinash, G., Nuttall, A. L., & Miller, J. M. (1992). The influence of loud sound on red blood cell velocity and blood vessel diameter in the cochlea. *Hearing Research*, 63(1–2), 102–107.

Quirk, W. S., Shivapuja, B. G., Schwimmer, C. L., & Seidman, M. D. (1994). Lipid peroxidation inhibitor attenuates noise-induced temporary threshold shifts. *Hearing Research*, 74(1–2), 217–220.

Rask-Andersen, H., Bostrom, M., Gerdin, B., Kinnefors, A., Nyberg, G., Engstrand, T., & Lindholm, D. (2005). Regeneration of human auditory nerve. In vitro/in video demonstration of neural progenitor cells in adult human and guinea pig spiral ganglion. *Hearing Research*, 203(1–2), 180–191.

Reyes, J. H., O'Shea, K. S., Wys, N. L., Velkey, J. M., Prieskorn, D. M., Wesolowski, K., & Altschuler, R. A. (2008). Glutamatergic neuronal differentiation of mouse embryonic stem cells after transient expression of neurogenin 1 and treatment with BDNF and GDNF: In vitro and in vivo studies. *The Journal of Neuroscience*, 28(48), 12622–12631.

Rivera, A., & Maxwell, S. A. (2005). The p53-induced gene-6 (proline oxidase) mediates apoptosis through a calcineurin-dependent pathway. *The Journal of Biological Chemistry*, 280(32), 29346–29354.

Roberts, R. A., Smith, R. A., Safe, S., Szabo, C., Tjalkens, R. B., & Robertson, F. M. (2010). Toxicological and pathophysiological roles of reactive oxygen and nitrogen species. *Toxicology*, 276(2), 85–94.

Ryals, B. M., & Rubel, E. W. (1988). Hair cell regeneration after acoustic trauma in adult Coturnix quail. *Science*, 240(4860), 1774–1776.

Salt, A. N., & Plontke, S. K. (2009). Principles of local drug delivery to the inner ear. *Audiology & Neuro-otology*, 14(6), 350–360.

Sato, Y., Kajiyama, S., Amano, A., Kondo, Y., Sasaki, T., Handa, S., & Ishigami, A. (2008). Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. *Biochemical & Biophysical Research Communications*, 375(3), 346–350.

Scheibe, F., Haupt, H., & Ising, H. (2000). Preventive effect of magnesium supplement on noise-induced hearing loss in the guinea pig. *European Archives of Oto-rhino-laryngology*, 257(1), 10–16.

Scheper, V., Paasche, G., Miller, J. M., Warnecke, A., Berkingali, N., Lenarz, T., & Stover, T. (2009). Effects of delayed treatment with combined GDNF and continuous electrical stimulation on spiral ganglion cell survival in deafened guinea pigs. *The Journal of Neuroscience Research*, 87(6), 1389–1399.

Schwartz, M. S., Otto, S. R., Shannon, R. V., Hitselberger, W. E., & Brackmann, D. E. (2008). Auditory brainstem implants. *Neurotherapeutics*, 5(1), 128–136.

Seidman, M. D., Shivapuja, B. G., & Quirk, W. S. (1993). The protective effects of allopurinol and superoxide dismutase on noise-induced cochlear damage. *Otolaryngology and Head and Neck Surgery*, 109(6), 1052–1056.

Seidman, M., Babu, S., Tang, W., Naem, E., & Quirk, W. S. (2003). Effects of resveratrol on acoustic trauma. *Otolaryngology and Head and Neck Surgery*, 129(5), 463–470.

Shah, S. B., Gladstone, H. B., Williams, H., Hradek, G. T., & Schindler, R. A. (1995). An extended study: Protective effects of nerve growth factor in neomycin-induced auditory neural degeneration. *The American Journal of Otology*, 16(3), 310–314.

Shibata, S. B., Cortez, S. R., Beyer, L. A., Wiler, J. A., Di Polo, A., Pfingst, B. E., & Raphael, Y. (2010). Transgenic BDNF induces nerve fiber regrowth into the auditory epithelium in deaf cochleae. *Experimental Neurology*, 223(2), 464–472.

Shim, H. J., Kang, H. H., Ahn, J. H., & Chung, J. W. (2009). Retinoic acid applied after noise exposure can recover the noise-induced hearing loss in mice. *Acta Oto-Laryngologica*, 129(3), 233–238.

Shoji, F., Miller, A. L., Mitchell, A., Yamasoba, T., Altschuler, R. A., & Miller, J. M. (2000a). Differential protective effects of neurotrophins in the attenuation of noise-induced hair cell loss. *Hearing Research*, 146(1–2), 134–142.

Shoji, F., Yamasoba, T., Magal, E., Dolan, D. F., Altschuler, R. A., & Miller, J. M. (2000b). Glial cell line-derived neurotrophic factor has a dose dependent influence on noise-induced hearing loss in the guinea pig cochlea. *Hearing Research*, 142(1–2), 41–55.

Spoendlin, H. (1984). Factors inducing retrograde degeneration of the cochlear nerve. *The Annals of Otology, Rhinology & Laryngology*, 112(Supplement), 76–82.

Spoendlin, H., & Schrott, A. (1990). Quantitative evaluation of the human cochlear nerve. *Acta Oto-Laryngologica*, 470(Supplementum), 61–69; discussion 69–70.

Staecker, H., Liu, W., Hartnick, C., Lefebvre, P., Malgrange, B., Moonen, G., & Van de Water, T. R. (1995). NT-3 combined with CNTF promotes survival of neurons in modiolus-spiral ganglion explants. *NeuroReport*, 6(11), 1533–1537.

Staecker, H., Kopke, R., Malgrange, B., Lefebvre, P., & Van de Water, T. R. (1996). NT-3 and/or BDNF therapy prevents loss of auditory neurons following loss of hair cells. *NeuroReport*, 7(4), 889–894.

Sugahara, K., Shimogori, H., & Yamashita, H. (2001). The role of acidic fibroblast growth factor in recovery of acoustic trauma. *NeuroReport*, 12(15), 3299–3302.

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126(4), 663–676.

Thorne, M., Salt, A. N., DeMott, J. E., Henson, M. M., Henson, O. W., Jr., & Gewalt, S. L. (1999). Cochlear fluid space dimensions for six species derived from reconstructions of three-dimensional magnetic resonance images. *The Laryngoscope*, 109(10), 1661–1668.

Tilstra, J., Rehman, K. K., Hennon, T., Plevy, S. E., Clemens, P., & Robbins, P. D. (2007). Protein transduction: Identification, characterization and optimization. *Biochemical Society Transactions*, 35(Pt 4), 811–815.

Ulfendahl, M. (2007). Tissue transplantation into the inner ear. In A. Martini, D. Stephens, & A. P. Read (Eds.), *Genes, Hearing and Deafness*. London: Martin Dunitz & Parthenon.

Ulfendahl, M., Hu, Z., Olivius, P., Duan, M., & Wei, D. (2007). A cell therapy approach to substitute neural elements in the inner ear. *Physiology & Behavior*, 92(1–2), 75–79.

Van de Water, T. R., Dinh, C. T., Vivero, R., Hoosien, G., Eshraghi, A. A., & Balkany, T. J. (2010). Mechanisms of hearing loss from trauma and inflammation: Otoprotective therapies from the laboratory to the clinic. *Acta Oto-Laryngologica*, 130(3),

Webster, M., & Webster, D. B. (1981). Spiral ganglion neuron loss following organ of Corti loss: A quantitative study. *Brain Research*, 212(1), 17–30.

Winkler, C., Kirik, D., & Bjorklund, A. (2005). Cell transplantation in Parkinson's disease: How can we make it work? *Trends in Neurosciences*, 28(2), 86–92.

Woodson, E. A., Reiss, L. A., Turner, C. W., Gfeller, K., & Gantz, B. J. (2010). The hybrid cochlear implant: A review. *Advances in Oto-Rhino-Laryngology*, 67, 125–134.

Yagi, M., Magal, E., Sheng, Z., Ang, K. A., & Raphael, Y. (1999). Hair cell protection from aminoglycoside ototoxicity by adenovirus-mediated overexpression of glial cell line-derived neurotrophic factor. *Human Gene Therapy*, 10(5), 813–823.

Yamagata, T., Miller, J. M., Ulfendahl, M., Olivius, N. P., Altschuler, R. A., Pyykko, I., & Bredberg, G. (2004). Delayed neurotrophic treatment preserves nerve survival and electrophysiological responsiveness in neomycin-deafened guinea pigs. *The Journal of Neuroscience Research*, 78(1), 75–86.

Yamashita, D., Jiang, H. Y., Schacht, J., & Miller, J. M. (2004). Delayed production of free radicals following noise exposure. *Brain Research*, 1019(1–2), 201–209.

Yamashita, D., Jiang, H. Y., Le Prell, C. G., Schacht, J., & Miller, J. M. (2005). Post-exposure treatment attenuates noise-induced hearing loss. *Neuroscience*, 134(2), 633–642.

Yamasoba, T., Nuttall, A. L., Harris, C., Raphael, Y., & Miller, J. M. (1998). Role of glutathione in protection against noise-induced hearing loss. *Brain Research*, 784(1–2), 82–90.

Yamasoba, T., Schacht, J., Shoji, F., & Miller, J. M. (1999). Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vivo. *Brain Research*, 815(2), 317–325.

Yamasoba, T., Altschuler, R. A., Raphael, Y., Miller, A. L., Shoji, F., & Miller, J. M. (2001). Absence of hair cell protection by exogenous FGF-1 and FGF-2 delivered to guinea pig cochlea in vivo. In D. Henderson, D. Prasher, R. Kopke, & R. Salvi (Eds.), *Noise Induced Hearing Loss: Basic mechanisms, prevention and control* (pp. 73–86). London: Noise in Network Publications.

Yamasoba, T., Pourbakht, A., Sakamoto, T., & Suzuki, M. (2005). Ebselen prevents noise-induced excitotoxicity and temporary threshold shift. *Neurosci Letters*, 380(3), 234–238.

Yeum, K. J., Beretta, G., Krinsky, N. I., Russell, R. M., & Aldini, G. (2009). Synergistic interactions of antioxidant nutrients in a biological model system. *Nutrition*, 25(7–8), 839–846.

Ylikoski, J., Pirvola, U., Moshnyakov, M., Palgi, J., Arumae, U., & Saarma, M. (1993). Expression patterns of neurotrophin and their receptor mRNAs in the rat inner ear. *Hearing Research*, 65(1–2), 69–78.

Ylikoski, J., Pirvola, U., Virkkala, J., Suvanto, P., Liang, X. Q., Magal, E., & Saarma, M. (1998). Guinea pig auditory neurons are protected by glial cell line-derived growth factor from degeneration after noise trauma. *Hearing Research*, 124(1–2), 17–26.

Yoshida, N., Kristiansen, A., & Liberman, M. C. (1999). Heat stress and protection from permanent acoustic injury in mice. *The Journal of Neuroscience*, 19(22), 10116–10124.

Zhai, S. Q., Wang, D. J., Wang, J. L., Han, D. Y., & Yang, W. Y. (2004). Basic fibroblast growth factor protects auditory neurons and hair cells from glutamate neurotoxicity and noise exposure. *Acta Oto-Laryngologica*, 124(2), 124–129.

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Effect of Vestibular Dysfunction on the Development of Gross Motor Function in Children with Profound Hearing Loss

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Key Words
Vestibular evoked myogenic potential · Caloric test · Rotational test · Gross motor development

Abstract
Objective: To evaluate the function of the superior and inferior vestibular nerve systems in children with profound sensorineural hearing loss, and to assess the influence of dysfunction of each vestibular nerve system on the development of gross motor function. ***Study Design:*** Retrospective study. ***Setting:*** A tertiary referral center. ***Methods:*** Eighty-nine children (age range: 20–97 months) with profound sensorineural hearing loss who were due to undergo cochlear implant surgery were recruited. Function of the superior vestibular nerve system was evaluated by the damped rotation test and the caloric test, whereas functions of the inferior vestibular nerve systems were evaluated by the vestibular evoked myogenic potential (VEMP) test. Gross motor development was assessed using the age of acquisition of head control and independent walking. ***Results:*** Among the children able to complete the vestibular function tests, abnormalities were found in 20% (16 of 84 children) in the damped rotation test, 41% (31 of 75 children) in the caloric test and 42% (26 of 62 children) in the VEMP test. Children who showed abnormal responses in the vestibular function tests showed significantly delayed acquisition of head control ($p < 0.05$) and independent walking ($p < 0.05$) in comparison with children with normal responses. The children who showed abnormal responses in all 3 vestibular tests showed the greatest delay in acquisition of gross motor function in comparison with the other groups. ***Conclusions:*** Children with profound hearing loss tend to have dysfunction in the superior as well as the inferior vestibular nerve systems. Both the superior and inferior vestibular nerve systems are important for the development of gross motor function in children.

Introduction

The development of balance and gross motor functions such as head control and independent walking are intimately related and dependent on inputs from the vestibular, visual, proprioceptive and motor systems [Kaga, 1999; Suarez et al., 2007]. During the early stages of development, children primarily depend on the visual system to maintain balance. As they grow older, they progressively begin to use somatosensory and vestibular information until these systems reach full maturity around the age of 10 years [Kaga, 1999; Wallacott et al., 2004;

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Suarez et al., 2007]. Since vestibular function plays an important role in the development of balance and locomotion, impairment of the vestibulospinal system in infancy may lead to delayed achievement of gross motor milestones [Eviatar et al., 1979; Kaga, 1999; Kaga et al., 2008].

A close relationship exists between the cochlea and the peripheral vestibular end organs with respect to embryology, physiology and anatomy [Jin et al., 2006; Cushing et al., 2008a], hence they may be similarly affected by embryological factors, or by viral or bacterial infections. Therefore, children with profound sensorineural hearing loss may also exhibit peripheral vestibular impairments [Shinjo et al., 2007; Cushing et al., 2008a; Kaga et al., 2008; Jacot et al., 2009]. It has been reported that children with profound hearing loss tend to display balance dysfunction and delayed acquisition of gross motor skills, such as head control, sitting and walking, compared with children with normal hearing [Potter and Silverman, 1984; Butterfield, 1986; Crowe and Horak, 1988; Suarez et al., 2007; Cushing et al., 2008a]. The incidence of vestibular dysfunction in children with profound hearing loss has been reported to be between 31 and 75% [Diepeveen and Jensen, 1968; Jin et al., 2006; Cushing et al., 2008a; Zagólski, 2008].

Several previous studies have investigated the relationship between vestibular function and gross motor development in children with profound hearing loss [Kaga et al., 1981; Potter and Silverman, 1984; Crowe and Horak, 1988; Suarez et al., 2007; Cushing et al., 2008a]. Kaga et al. [1981] showed that the age of acquiring head control and independent walking in children with vestibular dysfunction was significantly delayed compared with normal controls [Kaga et al., 1981]. Rine et al. [2000] reported delayed gross motor development in children with vestibular dysfunction. In these studies, vestibular function in infants and children was evaluated using the rotational test and/or the caloric test, which reflect function in the lateral semicircular canal and superior vestibular nerves [Kaga, 1999; Suarez et al., 2007; Cushing et al., 2008a]. Vestibular evoked myogenic potentials (VEMPs) in response to air-conducted sound have recently been used to evaluate vestibular function [Colebatch and Halmagyi, 1992; Murofushi et al., 1996, 1998; Welgampola and Colebatch, 2005]. Physiological and clinical studies have suggested that VEMPs are generated by activation of the saccule and the inferior vestibular nerves [McCue and Guinan, 1994; Murofushi et al., 1995, 1996]. Combined use of VEMP and rotational and/or caloric tests has enabled examination of the inferior and superior vestibular nerve systems separately [Murofushi et al., 1996, 1998; Iwasaki et al., 2005]. Although VEMPs have been studied mainly in adults, it has been shown that the VEMP can be recorded from infants and children in almost the same way as adults [Kelsch et al., 2006].

In the present study, we assessed vestibular function in children with profound hearing loss, before they underwent cochlear implantation, using VEMPs as well as caloric and rotational testing, and compared the results with the development of gross motor function. The purposes of the present study were to evaluate the function of the superior and inferior vestibular nerve systems in children with profound hearing loss, and to investigate the effect of each vestibular nerve system on the development of gross motor function.

Methods

We enrolled 101 consecutive new children (20–97 months old) who presented at the University of Tokyo Hospital between January 2003 and June 2010 with profound hearing loss and who subsequently underwent cochlear implant surgery. We excluded 11 children with acquired hearing loss (4 due to meningitis, 2 due to severe neonatal infections, and 5 who had passed the newborn hearing screening test). We also excluded 1 child with extremely low birth weight. We did not exclude 8 children with cytomegalovirus (CMV) infection or 2 with Waardenburg syndrome because they did not show any neurological abnormalities except for hearing loss. As a result, 89 children (45 male, 44 female; age range 20–97 months, mean age 40 months) were included. The data of individual children are listed in the online supplementary table 1 (see www.karger.com/doi/10.1159/000346344 for all online suppl. material). All these patients underwent high-resolution computed tomography of the temporal bone and magnetic resonance imaging of the brain. Screening for the connexin 26 *(GJB2)* mutation in the peripheral blood and screening for CMV DNA in the umbilical cord blood were performed in 31 and 28 patients, respectively. The etiologies of hearing loss in the 89 children are listed in table 1. Classification of the inner ear malformation [Sennaroglu and Saatci, 2002] in 19 patients is listed in the online supplementary table 2.

This study was approved by the ethics committee in the Faculty of Medicine at the University of Tokyo and was conducted according to the tenets of the Declaration of Helsinki. Written informed consent was obtained from the parents of each participant.

Evaluation of the Etiologies of Hearing Loss

High-resolution computed tomographic scans (slice thickness 1.0 mm), screening for CMV DNA in the umbilical cord blood, and screening for the *GJB2* mutation were performed. High-resolution computed tomographic scans were checked by both otolaryngologists and radiologists. Both homozygous and heterozygous mutations of *GJB2* were classified as positive. There was no patient selection protocol with regard to the performance of these tests, which may cause a bias in the etiology results. A family history of significant perinatal problems was checked by having parents complete questionnaires (open-ended questions). These procedures were approved by the local ethics committee.

Table 1. Etiologies of hearing loss in 89 children

	Number %	Mean age at evaluation, months
Inner ear malformation	19 (21%)	43 (25–87)
GJB2 mutation	13 (15%)	27 (20–33)
Congenital CMV infection	8 (9%)	38 (24–63)
Waardenburg syndrome	2 (2%)	51 (27–75)
Unknown	47 (54%)	42 (24–97)
Total	89 (100%)	40 (20–97)

Age ranges are indicated in parentheses.

Vestibular Function Tests

Damped Rotation Test

The children were held upright on their mother's knees on a rotational chair with their heads bending down 30°. The rotational chair was accelerated to a maximum rotational velocity of 200°/s with a maximum acceleration of 300°/s^2 and then decayed to 0°/s by a deceleration of −4°/s^2. The test was conducted twice in both clockwise and counterclockwise directions. Eye movements were recorded by electronystagmography. Since calibration for accurate velocity measurements could not be performed in most children, we calculated the number of beats of per-rotatory nystagmus. The number of beats was measured and compared with age-matched controls according to the results of the damped rotation test in normal children reported by Kaga et al. [1981] for children up to 6 years old. If the number of per-rotatory nystagmus beats was more than 2 standard deviations smaller than the average value at each age, as reported by Kaga et al. [1981], it was considered abnormal. For children older than 6 years, the normal limit of the number of per-rotatory nystagmus beats was set as 23. This value is based on the number of per-rotatory nystagmus beats in 15 normal children between the ages of 7 and 9 years (31 ± 3.9 beats) recorded in this laboratory.

Caloric Test

The caloric test was performed using 4°C ice water. Horizontal and vertical eye movements were recorded using electronystagmography. We measured the duration of induced nystagmus and compared it with age-matched controls since calibration of eye movements was difficult in most children. The duration of induced nystagmus in 112 normal control children was 94.7 ± 20.7 s for the age range 13–24 months, 103.8 ± 28.4 s for 25–36 months, 109.2 ± 28.4 s for 37–48 months, 98.1 ± 20.3 s for 49–60 months, 105 ± 28.4 for 61–72 months, and 123.3 ± 35.1 s for >72 months. If the duration of induced nystagmus was more than 2 standard deviations smaller than the average value at each age, it was considered abnormal. Therefore, normal limits were set as 53.3 s for 13–24 months, 54.3 s for 25–36 months, 52.4 s for 37–48 months, 57.4 s for 49–60 months, 48.1 s for 61–72 months and 35.1 s for >72 months.

Vestibular Evoked Myogenic Potentials

Each subject was placed in the supine position. The active electrode was placed over the upper half of the sternocleidomastoid muscle (SCM), the reference electrode on the upper sternum and the ground electrode on the midline of the forehead. Subjects were instructed to raise their heads off the pillow to activate the SCM. In children who could not follow this instruction, the examiner helped them to raise their body with their head hanging down to induce contraction of the SCM. Electromyographic activity in the SCMs was monitored to confirm sufficient normal muscle activity (>150 μV). Sound stimuli of 500-Hz tone bursts (95 dB nHL) were presented to each ear through calibrated headphones (DR-531, Elega Acoustic Co. Ltd., Tokyo, Japan). Electromyographic signals from the SCM on the stimulated side were amplified using Neuropack Sigma (Nihon Koden, Tokyo, Japan). The stimulation rate was 5 Hz, the band-pass filter intensity was 20–2000 Hz, and the analysis time was 50 ms. VEMPs in response to 50 stimuli were averaged twice. VEMPs were considered to be present when there was a reproducible short-latency biphasic wave (p13–n23) [Sheykholeslami et al., 2005; Kelsch et al., 2006]. We calculated the asymmetry ratio for the amplitude of VEMPs (VEMP AR) with the following formula using the peak-to-peak amplitude of p13–n23 (μV) on the right side *(Ar)* and that on the left side *(Al)*:

$$\text{VEMP AR (\%)} = 100 \cdot |(Ar - Al)/(Ar + Al)|.$$

VEMP AR (%) <33.3 was considered to indicate a significant asymmetry [Jin et al., 2006; Shinjo et al., 2007].

Gross Motor Development

To assess gross motor development, we interviewed parents about the age at which the children started to acquire head control and to walk by themselves. We also checked the ages given against the relevant data recorded in the Maternity Health Record Book provided by the Japanese government.

If the age of acquiring head control was >5 months and the age of independent walking was >18 months, the development of the gross motor function was considered to be delayed according to the modified version of DENVER II for Japanese children published by the Japanese Society of Child Health (Nihon Shoni Iji Shuppansha, Tokyo, Japan).

Statistics

For comparison of two groups, the Mann-Whitney U test was used. For comparing multiple groups, the nonparametric Kruskal-Wallis test was used. Variables that showed a significant difference in this test were then compared in pairs using the nonparametric Steel-Dwass multiple-comparison method. Values were expressed as means ± SD. A p value <0.05 was considered significant.

Results

Vestibular Function in Children with Profound Hearing Loss

A summary of the results of the damped rotation test, caloric test and VEMPs in the children with profound hearing loss is shown in table 2. Since these vestibular tests need a certain amount of cooperation, they could not be completed in some children. Among the 89 children recruited, 51 were able to complete all 3 vestibular tests

Table 2. Results of vestibular function testing

	Normal	Unilateral dysfunction	Bilateral dysfunction	Total
Inner ear malformation				
Rotation test	9 (50%)	0 (0%)	9 (50%)	18 (100%)
Caloric test	4 (27%)	4 (27%)	7 (47%)	15 (100%)
VEMP	6 (40%)	2 (13%)	7 (47%)	15 (100%)
GJB2 mutation				
Rotation test	13 (100%)	0 (0%)	0 (0%)	13 (100%)
Caloric test	13 (100%)	0 (0%)	0 (0%)	13 (100%)
VEMP	10 (83%)	0 (0%)	2 (17%)	12 (100%)
Congenital CMV infection				
Rotation test	3 (60%)	1 (20%)	1 (20%)	5 (100%)
Caloric test	4 (67%)	1 (17%)	1 (17%)	6 (100%)
VEMP	2 (33%)	1 (17%)	3 (50%)	6 (100%)
Others				
Rotation test	42 (88%)	0 (0%)	6 (13%)	48 (100%)
Caloric test	23 (56%)	8 (20%)	10 (24%)	41 (100%)
VEMP	18 (62%)	2 (7%)	9 (31%)	29 (100%)
All children				
Rotation test	67 (80%)	1 (1%)	16 (19%)	84 (100%)
Caloric test	44 (59%)	13 (17%)	18 (24%)	75 (100%)
VEMP	36 (58%)	5 (8%)	21 (34%)	62 (100%)

Table 3. Relationship between superior and inferior vestibular function tests

	Rotation test/caloric test			Total
	normal	asymmetry	bilateral dysfunction	
VEMP				
Normal	26	7	1	36
Asymmetry	1	3	1	5
Bilateral dysfunction	9	0	11	20
Total	36	10	15	61

whereas the other 38 children were only able to complete 1 or 2 of the tests.

The damped rotation test was completed in 84 of the 89 children (94%). Among these 84 children, 16 (19%) showed reduced or absent per-rotatory nystagmus on both clockwise and counterclockwise rotations, whereas 67 children (80%) showed normal responses during rotation in both directions. One child (1%) showed reduced per-rotatory nystagmus in the clockwise rotations only (patient No. 36 in the online suppl. table 1).

Caloric testing was completed in 75 of the 89 children (84%). Among them, 18 children (24%) showed reduced or absent nystagmus induced in both ears, whereas 44 children (59%) showed normal responses in both ears. Thirteen children (17%) showed abnormal responses in one ear only.

VEMP testing was completed in 62 of the 89 children (70%). Among them, 21 children (34%) showed no responses on either side whereas 36 children (58%) showed normal responses on both sides. Five children (8%) showed responses on one side only.

Most children with the *GJB2* mutation showed normal responses bilaterally in the damped rotation test, caloric test and VEMP test. On the other hand, more than half of the children with inner ear malformations and congenital CMV infection showed abnormal responses in these 3 vestibular function tests (table 2).

The relationship between the results of the damped rotation test, caloric test and VEMP test are shown in table 3. Since both the damped rotation and caloric tests reflect the function of the superior vestibular nerve system, we combined the results of these two tests. If cases showed abnormal responses in either of these two tests, we classified them as having abnormal superior vestibular function. Cases which showed abnormal VEMP responses were classified as having abnormal inferior vestibular function. Among the 61 children who were able to complete all 3 vestibular tests, 26 (43%) showed normal responses in both the superior and inferior vestibular function tests whereas 15 children (25%) showed abnormal responses in both of these tests. Ten children (16%) showed abnormalities in the superior vestibular function tests while sparing inferior vestibular function. On the other hand, 10 children (16%) showed abnormalities in the inferior vestibular dysfunction tests while sparing superior vestibular nerve function.

Gross Motor Development in Children with Profound Hearing Loss

The distribution of ages at which children with profound hearing loss started acquiring head control and independent walking are shown in figure 1 and table 4. We were unable to obtain information regarding the age of acquisition of head control in 10 children, and the age of independent walking in 13 children.

The age at which the children acquired head control was delayed to later than 5 months of age in 24 (30%) of 79 children. The age at which children began to walk independently was delayed to later than 18 months of age in 20 (26%) of 76 children.

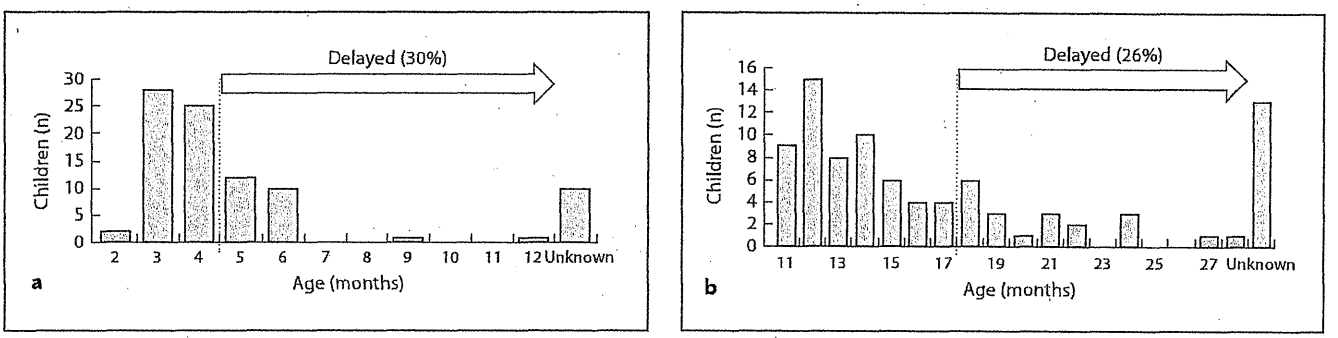


Fig. 1. Distribution of the age of acquiring head control and independent walking in children with profound hearing loss. **a** Distribution of the age of acquiring head control. Ages greater than 5 months were considered to be delayed. **b** Distribution of the age of independent walking. Ages greater than 18 months were considered to be delayed.

Table 4. Age of acquiring head control and independent walking

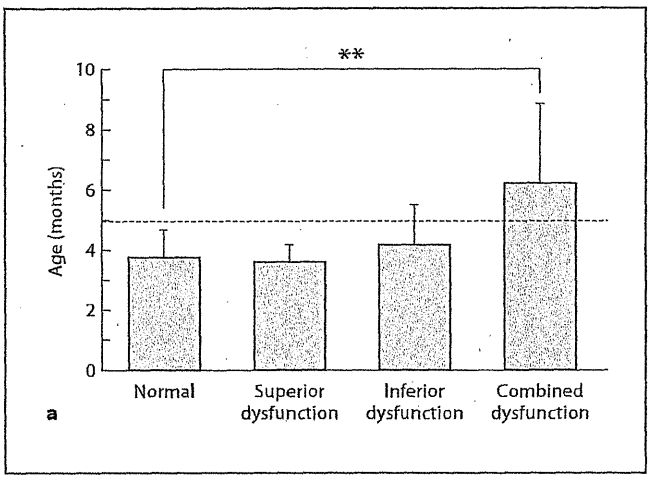
	Head control			Independent walking			Total
	normal	delayed	unknown	normal	delayed	unknown	
Inner ear malformation	8 (42%)	8 (42%)	3 (16%)	8 (42%)	7 (37%)	4 (21%)	19 (100%)
GJB2 mutation	11 (85%)	1 (8%)	1 (8%)	13 (100%)	0	0	13 (100%)
Congenital CMV infection	6 (50%)	1 (13%)	1 (13%)	4 (50%)	2 (25%)	2 (25%)	8 (100%)
Others	30 (4%)	14 (29%)	5 (10%)	31 (63%)	11 (22%)	7 (14%)	49 (100%)
All children	55 (62%)	24 (27%)	10 (11%)	56 (33%)	20 (22%)	13 (15%)	89 (100%)

In most children with the *GJB2* mutation, the age of acquiring head control and independent walking was within normal limits (11 of 12 children with available information for head control; all children with available information for independent walking; table 4). On the other hand, approximately half of the children with inner ear malformations showed delayed head control (8 of 16 children) and delayed independent walking (7 of 15 children). In children with CMV infection, the age of independent walking was delayed in one third (2 of 6 children) whereas the age of acquiring head control was delayed in one seventh of them (1 of the 7 children).

Vestibular Function and the Development of Gross Motor Function

To estimate the effect of vestibular dysfunction on the development of gross motor function, we compared the age of acquiring head control and independent walking with the results of each of the vestibular function tests (table 5). The age of acquiring both head control and independent walking was significantly delayed in children who showed abnormal responses bilaterally in comparison with those who showed normal responses bilaterally ($p < 0.05$ in the rotation test, caloric test and VEMP test for both head control and independent walking). On the other hand, there were no significant differences in the age of acquiring head control and independent walking between the children who showed asymmetric responses and those with normal responses in the caloric and VEMP testing ($p > 0.05$ for both tests).

To clarify the effect of dysfunction of the superior and inferior vestibular nerve systems on the development of gross motor function, we classified the children according to the involvement of the superior and the inferior vestibular nerve systems into the following 4 groups: (1) normal group, i.e. children who showed normal responses bilaterally in both the superior vestibular function tests (caloric testing and damped rotation test) and the inferior vestibular function test (VEMPs) (n = 26); (2) superior dysfunction group, i.e. those with abnormal re-



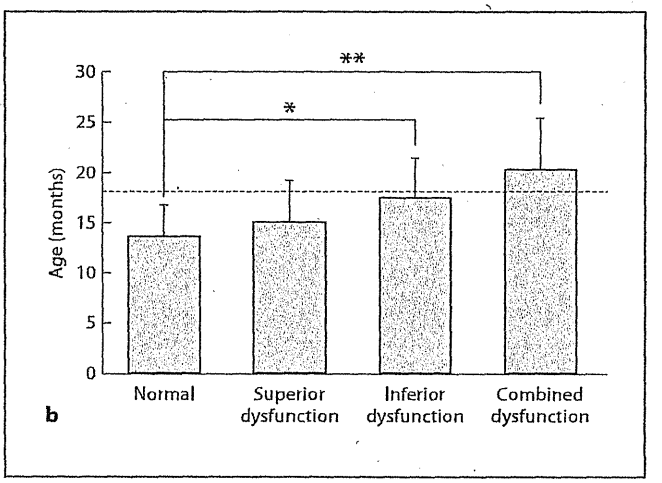


Fig. 2. Comparison of the age of acquiring head control and independent walking in children with profound hearing loss classified according to the involvement of the superior and inferior vestibular nerve systems. * $p < 0.05$, ** $p < 0.01$. **a** The age of acquiring head control in children with normal vestibular function, those with involvement of the superior vestibular nerve system sparing the inferior vestibular nerve system (superior dysfunction), those with involvement of the inferior vestibular nerve system sparing the superior vestibular nerve system (inferior dysfunction) and those with involvement of both the superior and inferior vestibular nerve systems (combined dysfunction). **b** The age of independent walking in the normal, superior, inferior and combined dysfunction groups.

Table 5. Age (months) of gross motor development in relation to vestibular function test results

	Normal	Asymmetry	Bilateral dysfunction
Head control			
Rotation test	3.8±1.1 (59)	3 (1)	5.6±2.3 (14)
Caloric test	3.7±1.0 (37)	4.0±1.0 (12)	5.2±2.2 (17)
VEMP	3.7±0.8 (31)	4.8±1.1 (5)	5.3±2.2 (19)
Independent walking			
Rotation test	14.6±3.5 (59)	14 (1)	19.1±4.8 (12)
Caloric test	14.5±3.8 (39)	14.0±2.1 (12)	18.4±5.0 (15)
VEMP	13.7±2.7 (29)	15.8±4.6 (4)	18.8±4.7 (17)

Data are shown as means ± SD; numbers of children are indicated in parentheses.

sponses bilaterally in either of the superior vestibular function tests in the presence of normal VEMP responses bilaterally (n = 9); (3) inferior dysfunction group, i.e. those with abnormal VEMP responses bilaterally in the presence of normal superior vestibular function tests (n = 3), and (4) combined dysfunction group, i.e. those with abnormal responses bilaterally in both the superior and inferior vestibular function tests (n = 11). Ten patients who showed unilateral vestibular dysfunction in either the damped rotation test or the caloric test (patients No. 12, 14, 15, 38, 50, 51, 57, 62, 64 and 69 in the online suppl. table 1) and 5 patients who showed abnormal VEMP responses on one side (patients No. 12, 14, 38, 77 and 83) were excluded from this analysis. Among them, 2 children (patients No. 14 and 38) showed dysfunction on the same sides in both caloric and VEMP tests, whereas the other children showed dysfunction on different sides in these tests. The age of acquiring head control was significantly delayed in the combined dysfunction group in comparison with the normal group ($p < 0.01$), whereas there were no significant differences among the normal group, the superior dysfunction group and the inferior dysfunction group ($p > 0.1$; fig. 2a). The age of independent walking was significantly delayed in the combined dysfunction group and in the inferior dysfunction group compared with the normal group ($p < 0.01$ and $p < 0.05$, respectively), whereas there were no significant differences between the normal group and the superior dysfunction group ($p > 0.8$) or between the inferior dysfunction group and the combined dysfunction group ($p > 0.5$; fig. 2b).

Discussion

In the present study, we have shown that approximately 40% of children with profound hearing loss have dysfunction of the superior vestibular nerve system, approximately 40% have dysfunction of the inferior vestibular nerve system, and approximately 20% have dysfunction of both vestibular nerve systems. Acquisition of head control and independent walking in children with bilateral vestibular dysfunction was significantly delayed in comparison with those with normal vestibular function.

In previous studies, vestibular function in infants and children has been evaluated by rotation and caloric tests, which reflect the function of the lateral semicircular canals and superior vestibular nerve [Diepeveen and Jensen, 1968; Kaga et al., 1981; Kaga, 1999; Buchmann et al., 2004; Shinjo et al., 2006; Jacot et al., 2009]. Kaga et al. [1981] examined vestibular function using the damped rotation test in 22 children with congenital deafness and found hypoactivity of the vestibulo-ocular reflexes in 12 children (55%). Shinjo et al. [2007] assessed vestibular function in 20 children with severe hearing loss using the damped rotation and caloric tests, and reported that abnormalities were found in 85% of these children with caloric testing and in 30% with the rotation test. Jacot et al. [2009] examined 224 children with profound hearing loss, using the caloric and rotation tests. They showed that 50% of the children tested have unilateral or bilateral vestibular dysfunction. In the present study, 41% of the children tested showed abnormal caloric responses and 20% showed abnormal responses in the damped rotation test. This prevalence of vestibular dysfunction was lower compared to that of previous studies [Kaga et al., 1981; Shinjo et al., 2007; Jacot et al., 2009]. This discrepancy might be caused by differences between the patient groups since the number of children with the *GJB2* mutation was relatively higher in our study compared to those previous studies [Buchman et al., 2004; Shinjo et al., 2007]. In the present study, most children with the *GJB2* mutation showed normal responses bilaterally in the damped rotation test, caloric test and with VEMPs. This result is consistent with previous reports showing that vestibular function is rarely affected in patients with the *GJB2* mutation [Todt et al., 2005; Tsukada et al., 2010]. On the other hand, more than half of the children with inner ear malformations and congenital CMV infection showed abnormal vestibular function in at least 1 of the 3 kinds of vestibular function tests used in the present study.

VEMPs in response to air-conducted sound have been used to evaluate vestibular function, especially that of the saccule and inferior vestibular afferents [Welgampola and Colebatch, 2005]. Combined use of VEMPs and the caloric test has enabled examination of the superior and inferior vestibular nerve systems separately [Murofushi et al., 1998; Iwasaki et al., 2005]. VEMPs have been extensively studied primarily in adult subjects since VEMPs require neck contraction during recording. However, several recent studies have shown that VEMPs can be recorded from infants and children in almost the same way as adults [Tribukait et al., 2004; Jin et al., 2006; Kelsh et al., 2006; Shinjo et al., 2007]. Tribukait et al. [2004] recorded VEMPs in 39 deaf children between the ages of 15 and 17 years and reported that VEMPs were absent bilaterally in 22% and asymmetric in 19%. Shinjo et al. [2007] recorded VEMPs in 20 children with profound deafness with ages ranging from 2 to 7 years and reported that 20% of patients showed no responses bilaterally and 30% showed asymmetric responses. In the present study, we attempted to record VEMPs from children by helping them to raise their heads during the recording. Furthermore, we used a 95-dB nHL tone burst instead of 90-dB nHL clicks, which were used in the study by Kelsch et al. [2006], as a stimulus for eliciting VEMPs, since it has been shown that tone bursts are superior to clicks in eliciting VEMP responses [Murofushi et al., 1999; Viciana and Lopez-Escamez, 2012]. Of the children tested in this study, with an age range of 2–8 years, 70% were able to generate sufficient neck muscle activity (>150 μV) to successfully complete VEMP testing. Among these children, 8% showed asymmetric VEMP responses and 34% showed no VEMP responses on either side, indicating that approximately 40% of these children with profound hearing loss have dysfunction of the inferior vestibular system on at least one side. This finding is compatible with the finding in previous studies in terms of the percentage of children showing inferior nerve system dysfunction [Tribukait et al., 2004; Shinjo et al., 2007].

In the present study, both the ages of acquiring head control and independent walking were significantly delayed in children with vestibular dysfunction in comparison with those with normal vestibular function. All the children were able to walk independently within 30 months. A few previous studies have shown that gross motor development is delayed in children with bilateral vestibular dysfunction [Kaga et al., 1981; Rine et al., 2000]. Kaga et al. [1981] reported that the age of acquiring head control and independent walking in children with bilateral vestibular dysfunction was significantly delayed

when compared with normal controls. They also reported that all children of preschool age with vestibular dysfunction were able to achieve head control, independent walking and running, suggesting the substitution of vestibular function by other sensory inputs such as visual and somatosensory cues [Kaga et al., 1981; Wallacott et al., 2004]. The development of gross motor function is affected by various factors including the functioning of the visual, vestibular, proprioceptive and motor systems [Kaga, 1999; Wallacott et al., 2004; Suarez et al., 2007]. It has been shown that a substantial proportion of children with profound hearing loss show balance dysfunction, especially when visual and/or somatosensory information is disturbed [Suarez et al., 2007; Cushing et al., 2008b]. Since the relative importance of visual, vestibular and somatosensory inputs to head stabilization and balance control has been shown to change dynamically during preschool ages [Berger et al., 1987; Assaiante and Ambrad, 1992], it is possible that the contribution of visual and somatosensory inputs steadily increases with age in children with vestibular dysfunction. Several studies have shown that children with bilateral vestibular dysfunction show postural instability in conditions with reduced visual and/or somatosensory cues [Enbom et al., 1991; Cushing et al., 2008b].

The contribution of the superior and inferior vestibular nerve systems to the development of gross motor function has not been studied previously. We classified children with profound hearing loss into 4 groups according to the results of 3 vestibular tests (normal function, superior dysfunction, inferior dysfunction, combined dysfunction) and compared the gross motor development among these groups. The age at acquisition of both head control and independent walking in the combined dysfunction group was the latest among the 4 groups, suggesting that the inferior as well as the superior nerve systems play an important role in gross motor development. Furthermore, the age of acquiring independent walking was significantly delayed in the inferior dysfunction group as well as the combined dysfunction group in comparison with the normal group, whereas it was not significantly different between the superior dysfunction group and the normal group. The inferior vestibular nerve system, which has an input to neck and leg muscles, may have a greater influence on the acquisition of independent walking than the superior vestibular nerve system.

In conclusion, we have shown that a substantial proportion of children with profound hearing loss have dysfunction of the inferior as well as the superior vestibular nerve system and that they show delayed acquisition of gross motor function. Since the development of gross motor function varies according to the extent of the involvement of each vestibular nerve system, it is preferable to evaluate both the superior and inferior vestibular function separately in order to form an individualized treatment plan for each child with profound hearing loss.

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Disclosure Statement

We have no conflicts of financial interest in this paper.

References

Assaiante C, Amblard B: Peripheral vision and age-related differences in dynamic balance. Hum Mov Sci 1992;11:533–548.

Berger W, Quintern J, Dietz V: Afferent and efferent control of stance and gait: developmental changes in children. Electroencephalogr Clin Neurophysiol 1987;66:244–252.

Buchman CA, Joy J, Hodges A, Telischi FF, Balkany TJ: Vestibular effects of cochlear implantation. Laryngoscope 2004;114:1–22.

Butterfield SA: Gross motor profiles of deaf children. Percept Mot Skills 1986;62:68–70.

Colebatch JG, Halmagyi GM: Vestibular evoked potentials in human neck muscles before and after unilateral vestibular deafferentation. Neurology 1992;42:1635–1636.

Crowe TK, Horak FB: Motor proficiency associated with vestibular deficits in children with hearing impairments. Phys Ther 1988;68:1493–1499.

Cushing SL, Chia R, James AL, Papsin BC, Gordon KA: A test of static and dynamic balance function in children with cochlear implants: the vestibular Olympics. Arch Otolaryngol Head Neck Surg 2008a;134:34–38.

Cushing SL, Papsin BC, Rutka JA, James AL, Gordon KA: Evidence of vestibular and balance dysfunction in children with profound sensorineural hearing loss using cochlear implants. Laryngoscope 2008b;118:1814–1823.

Diepeveen JE, Jensen J: Differential caloric reactions in deaf children. Acta Otolaryngol 1968;65:570–574.

Enbom H, Magnusson M, Pyykko I: Postural compensation in children with congenital or early acquired bilateral vestibular loss. Ann Otol Rhinol Laryngol 1991;100:472–478.

Eviatar L, Miranda S, Eviatar A, Freeman K, Borkowski M: Development of nystagmus in response to vestibular stimulation in infants. Ann Neurol 1979;5:508–514.

Iwasaki S, Takai Y, Ito K, Murofushi T: Abnormal vestibular evoked myogenic potentials in the presence of normal caloric responses. Otol Neurotol 2005;26:1196–1199.

Jacot E, van den Abbeele T, Debre HR, Wiener-Vacher SR: Vestibular impairments pre- and post-cochlear implant in children. Int J Pediatr Otorhinolaryngol 2009;73:209–217.
Jin Y, Nakamura M, Shinjo Y, Kaga K: Vestibular-evoked myogenic potentials in cochlear implant children. Acta Otolaryngol 2006;126: 164–169.
Kaga K: Vestibular compensation in infants and children with congenital and acquired vestibular loss in both ears. Int J Pediatr Otorhinolaryngol 1999;49:215–224.
Kaga K, Shinjo Y, Jin Y, Takegoshi H: Vestibular failure in children with congenital deafness. Int J Audiol 2008;47:590–599.
Kaga K, Suzuki JI, Marsh RR, Tanaka Y: Influence of labyrinthine hypoactivity on gross motor development of infants. Ann NY Acad Sci 1981;374:412–420.
Kelsch TA, Schaefer LA, Esquivel CR: Vestibular evoked myogenic potentials in young children: test parameters and normative data. Laryngoscope 2006;116:895–900.
McCue MP, Guinan JJ: Acoustically responsive fibers in the vestibular nerve of the cat. J Neurosci 1994;14: 6058–6070.
Murofushi T, Curthoys IS, Topple AN, Colebatch JG, Halmagyi GM: Responses of guinea pig primary vestibular neurons to clicks. Exp Brain Res 1995;103:174–178.
Murofushi T, Halmagyi GM, Yavor RA, Colebatch JG: Absent vestibular evoked myogenic potentials in vestibular neurolabyrinthitis. An indicator of inferior vestibular nerve involvement? Arch Otolaryngol Head Neck Surg 1996;122:845–848.
Murofushi T, Matsuzaki M, Mizuno M: Vestibular evoked myogenic potentials in patients with acoustic neuromas. Arch Otolaryngol Head Neck Surg 1998;124:509–512.
Murofushi T, Matsuzaki M, Wu CH: Short tone burst-evoked myogenic potentials on the sternocleidomastoid muscle: are these potentials also of vestibular origin? Arch Otolaryngol Head Neck Surg 1999;125:660–664.
Potter CN, Silverman LN: Characteristics of vestibular function and static balance skills in deaf children. Phys Ther 1984;64:1071–1075.
Rine RM, Cornwall G, Gan K, LoCascio C, O'Hare T, Robinson E, Rice M: Evidence of progressive delay of motor development in children with sensorineural hearing loss and concurrent vestibular dysfunction. Percept Mot Skills 2000;90:1101–1112.
Sennaroglu L, Saatci I: A new classification for cochleovestibular malformations. Laryngoscope 2002;112:2230–2241.
Sheykholeslami K, Megerian CA, Arnold JE, Kaga K: Vestibular-evoked myogenic potentials in infancy and early childhood. Laryngoscope 2005;115:1440–1444.
Shinjo Y, Jin Y, Kaga K: Assessment of vestibular function of infants and children with congenital and acquired deafness using the ice-water caloric test, rotational chair test and vestibular-evoked myogenic potential recording. Acta Otolaryngol 2007;127:736–747.
Suarez H, Angeli S, Suarez A, Rosales B, Carrera X, Alonso R: Balance sensory organization in children with profound hearing loss and cochlear implants. Int J Pediatr Otorhinolaryngol 2007;71:629–637.
Todt I, Hennies HC, Basta D, Ernst A: Vestibular dysfunction of patients with mutations of connexin 26. Neuroreport 2005;16:1179–1181.
Tribukait A, Brantberg K, Bergenius J: Function of semicircular canals, utricle and saccules in deaf children. Acta Otolaryngol 2004;124:41–48.
Tsukada K, Nishio S, Usami S, Deafness Gene Study Consortium: A large cohort study of *GJB2* mutations in Japanese hearing loss patients. Clin Genet 2010;78:464–470.
Viciana D, Lopez-Escamez JA: Short tone bursts are better than clicks for cervical vestibular-evoked myogenic potentials in clinical practice. Eur Arch Otorhinolaryngol 2012;269: 1857–1863.
Wallacott MH, Assaiante C, Amblard B: Development of balance and gait control; in Bronstein AM, Brandt T, Wallacott MH, Nutt JG (eds): Clinical Disorders of Balance, Posture and Gait, ed 2. London, Arnold, 2004, pp 39–62.
Welgampola MS, Colebatch JG: Characteristics and clinical applications of vestibular-evoked myogenic potentials. Neurology 2005;64: 1682–1688.
Zagólski O: Vestibular-evoked myogenic potentials and caloric stimulation in infants with congenital cytomegalovirus infection. J Laryngol Otol 2008;122:574–579.

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REGULAR ARTICLE

Developmental changes in the responsiveness of rat spiral ganglion neurons to neurotrophic factors in dissociated culture: differential responses for survival, neuritogenesis and neuronal morphology

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Abstract The way that the development of the inner ear innervation is regulated by various neurotrophic factors and/or their combinations at different postnatal developmental stages remains largely unclear. Moreover, survival and neuritogenesis in deafferented adult neurons is important for cochlear implant function. To address these issues, developmental changes in the responsiveness of postnatal rat spiral ganglion neurons (SGNs) to neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and leukemia inhibitory factor (LIF) were examined by using a dissociated cell culture system. SGNs at postnatal day (P) 0, P5 and P20 (young adult) were cultured with the addition of NT-3, BDNF, or LIF or of a combination of NT-3 and BDNF (N + B) or of NT-3, BDNF and LIF (ALL factors). SGNs were analyzed for three parameters: survival, longest neurite length (LNL) and neuronal morphology. At P0, SGNs required exposure to N + B or ALL factors for enhanced survival and the ALL factors combination showed a synergistic effect much greater than the sum of the individual factors. At P5, SGNs responded to a wider range of treatment conditions for enhanced survival and combinations showed only an additive improvement over individual factors. The survival percentage of untreated SGNs was highest at P20 but combinations of neurotrophic factors were no more effective than individual factors. LNL of each SGN was enhanced by LIF alone or ALL factors at P0 and P5 but was suppressed by NT-3, BDNF and N + B at P5 in a dose-dependent manner. The LNL at P20 was enhanced by ALL factors and suppressed by N + B. Treatment with ALL factors increased the proportion of SGNs that had two or more primary neurites in all age groups. These findings suggest that NT-3, BDNF, LIF and their combinations predominantly support different ontogenetic events at different developmental stages in the innervation of the inner ear.

Keywords NT-3 · BDNF · LIF · Age-related changes · Inner ear · Rat (Sprague Dawley)

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Introduction

In the auditory system, various growth factors are known to play roles in the development of the innervation of the inner

ear (Fritzsch et al. 1997b, 2004; Rubel and Fritzsch 2002). For example, studies of transgenic mice have shown that two members of the nerve growth factor (NGF) family of proteins, namely brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), play essential roles in the normal development of innervation (Agerman et al. 2003; Ernfors et al. 1995; Farinas et al. 2001; Fritzsch et al. 1997a, 2004; Tessarollo et al. 2004). In vitro studies of spiral ganglion neurons (SGNs) have revealed that both NT-3 and BDNF can promote SGN survival and neuritogenesis in organotypic SG explants (Aletsee et al. 2001; Hartnick et al. 1996; Mou et al. 1997; Mullen et al. 2012; Pirvola et al. 1992) and in dissociated cultures (Gillespie et al. 2001; Hartnick et al. 1996; Hegarty et al. 1997; Malgrange et al. 1996; Marzella et al. 1999; Pirvola et al. 1994; Vieira et al. 2007; Wei et al. 2007; Whitlon et al. 2006; Zheng et al. 1995). Recent studies have shown that neurotrophins also regulate the physiological properties of SGNs (Adamson et al. 2002; Davis and Liu 2011). Other peptide growth factors, such as ciliary-derived neurotrophic factor and leukemia inhibitory factor (LIF), also promote survival and neuritogenesis in SGNs in vitro (Gillespie et al. 2001; Hartnick et al. 1996; Marzella et al. 1997; Vieira et al. 2007; Whitlon et al. 2006).

In various peripheral nervous systems, the magnitude and manner of the neuron's response to neurotrophic factors is known to be age-dependent. For example, sympathetic neurons require NGF for their survival in early development. After they become mature, they are less dependent on NGF for survival (Easton et al. 1997; Putcha et al. 2000) but still respond to NGF with increased neurite growth (Orike et al. 2001b). Moreover, the survival dependence of trigeminal ganglion neurons has been shown to switch from BDNF and NT-3 to NGF during the early stages of target field innervation (Buchman and Davies 1993). Similarly, a subpopulation of dorsal root ganglion (DRG) neurons depends on NGF during embryonic development but switches its dependence to glial-cell-derived neurotrophic factor in early postnatal life (Molliver et al. 1997). These data suggest that various neurotrophic factors can exert their effects on the same neuronal population, either simultaneously or sequentially, at different phases of their development and further, that each factor might regulate different ontogenetic events at different developmental stages. Although a few studies have addressed this issue in the vestibular system (Chihara et al. 2011; Hashino et al. 1999), the degree to which similar age-dependent regulation of SGNs by neurotrophic factors occurs remains unclear.

Accordingly, we have performed a systematic developmental study of rat SGNs by culturing dissociated SGNs harvested at various postnatal stages from birth to the age at which the hearing function is mature. We have tested the responsiveness of these SGNs to NT-3, BDNF and LIF, alone or in various combinations, focusing mainly on SGN survival, neuritogenesis and the neuronal morphology of individual SGNs.

Materials and methods

All animal procedures were approved by the relevant local animal subjects committees (Graduate School of Medicine, The University of Tokyo, #P08-029) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preparation of tissue culture plates

Circular coverslips (13 mm; Matsunami glass, Tokyo, Japan) were incubated with a mixed solution of fibronectin (Sigma Aldrich Japan, Tokyo, Japan; 10 μg/ml in phosphate-buffered saline [PBS; pH 7.4]) and laminin (Sigma Aldrich Japan; 10 μg/ml in PBS) at 4°C overnight and then washed three times in PBS (pH 7.4). The coverslips were then incubated with poly-L-lysine (Sigma Aldrich Japan; 20 μg/ml in Dulbecco's modified Eagle's medium [DMEM; Invitrogen life technologies, Tokyo Japan]) at 37°C for 1 h, washed three times in PBS (pH 7.4), air-dried and placed in each well of 24-well plates (BD Biosciences, Tokyo, Japan).

Animal dissection and dissociated cell culture of SGNs

Cultures of dissociated SGNs were prepared from Sprague Dawley rats (Saitama experimental animals, Saitama, Japan) at postnatal day 0 (P0), day 5 (P5) and day 20 (P20). We chose these developmental ages for the following reason: the period of P0 culture (equivalent to P0-P4 in vivo) corresponds to the time of naturally occurring SGN death (Echteler et al. 2005; Rueda et al. 1987); P5 culture (equivalent to P5–P9 in vivo) corresponds to the terminal stage of the remodeling of afferent projections within the sensory epithelium (Echteler 1992; Wiechers et al. 1999); and P20 culture (equivalent to P20–P24 in vivo) corresponds to the stage in which all of the major developmental events for SGNs are complete and the hearing function has achieved maturity (Pujol et al. 1998).

For the dissociation of SGNs, rats were deeply anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (9 mg/kg) and then decapitated. The mandible was removed and skulls were opened mid-sagitally. Under a dissecting microscope, the brain was removed and the temporal bones were harvested and transferred to Petri dishes containing sterile PBS (pH 7.4). The membranous labyrinth was exposed by removal of the bony or cartilaginous cochlear capsule. After

the removal of the spiral ligament, stria vascularis and organ of Corti, the SG was carefully dissected from the modiolus by using fine forceps and placed in calcium/magnesium-free Hank's balanced salt solution (HBSS, Invitrogen life technologies). Pooled ganglia (n=10–16) were then enzymatically digested in HBSS with 0.1% collagenese and then in HBSS with 0.25% trypsin (both from Invitrogen life technologies) in a gently shaking 37°C water bath. Incubation times varied depending on the age of the tissue (see Table 1). Enzymatic digestion was terminated by the addition of fetal bovine serum (Invitrogen life technologies) to a final concentration of 10% and the digested ganglia were centrifuged at 1,500 rpm for 5 min at room temperature (RT). The supernatant was discarded and fresh primary growth medium consisting in DMEM (Invitrogen life technologies), fetal bovine serum (10%; Invitrogen life technologies), HEPES buffer (10 mM; Invitrogen life technologies) and penicillin (300 U/ml; Sigma Aldrich Japan) was then added, following which the ganglia were dissociated by mechanical trituration through a plastic pipette tip. In this procedure, a maximum of four triturations were carried out before the cells were removed by decanting the supernatant medium. Fresh medium was added, a further four triturations were carried out and the cells were again removed. These sequential triturations were repeated until the majority of the neurons were removed, with the number of repeats depending on the age of the donor. This method of avoiding excess mechanical damage to the dissociated SGNs enhanced the survival of SGNs several fold, especially in P20 SGNs, as demonstrated in the dissociation of superior cervical ganglion neurons (Orike et al. 2001a).

Each cell suspension was again spun at 1,500 rpm for 5 min and the resultant pellet was resuspended in fresh primary growth medium. Cell counts were made by using a hemocytometer and dissociated cells were plated onto the center of the glass coverslips at a density of 20,000 cells/100 μl per well. The cultures were maintained at 37°C in a humidified atmosphere containing 8% CO_2 for 2 h to promote the attachment of the neurons to the bottom of the coverslips. The cultures were then incubated in 300 μl primary growth medium for 10 h for the complete attachment of the SGNs, followed by incubation in a serum-free maintenance medium consisting in DMEM, N2 supplement (10 μl/ml; Invitrogen life technologies), HEPES buffer (10 mM), penicillin (300 U/ml), glucose (at a final concentration of 6 g/l; Sigma Aldrich Japan) for an additional 72 h. To compare the effects of neurotrophic factors, cultures in each age group were further divided into six subgroups and supplemented with the following factors: (1) recombinant human NT-3 (catalog number 480875; EMD biosciences, La Jolla, Calif., USA; 50 ng/ml), (2) recombinant human BDNF (catalog number 01-194; Upstate Biotechnology, Lake Placid, N.Y., USA; 50 ng/ml), (3) LIF (catalog number LIF3005; Chemicon, Temecula, Calif., USA; 50 ng/ml), (4) a combination of NT-3 and BDNF (50 ng/ml each; referred to as the N + B group), (5) a combination of NT-3, BDNF and LIF (50 ng/ml each; referred to as the ALL factor group). One group was cultured without addition of any of the factors to serve as an untreated control. The concentration of each factor was determined based on previous studies that had examined the responses of SGNs from postnatal mice and rats in vitro (Gillespie et al. 2001; Hegarty et al. 1997; Malgrange et al. 1996; Marzella et al. 1999; Vieira et al. 2007; Wei et al. 2007; Whitlon et al. 2006). To assess the effects of concentration on SGN neurite extension (see Results), additional P5 cultures were treated with NT-3 (10 ng/ml), BDNF (10 ng/ml), or N + B (10 ng/ml each).

To determine the initial yields of plated SGNs, cultures (n=4 in each age group) were maintained in primary growth medium for 4 h after plating, followed by fixation for immunostaining.

Table 1 Conditions for dissociation of spiral ganglion neurons (SGNs) at postnatal day 0 (P0), P5 and P20

Treatment	P0	P5	P20
Duration of collagenase incubation (min)	15	20	30
Duration of trypsin incubation (min)	20	30	40

Immunohistochemistry

Cultured cells were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at RT. After several PBS (pH7.4) washes, endogeneous peroxidase activity was blocked by treatment with 0.3% hydrogen peroxide in methanol for 30 min at RT. The cells were then incubated with blocking solution (PBS, pH 7.4, containing 4% fetal bovine serum [Invitrogen life technologies], 0.2% Triton X-100 and 0.1% sodium azide) for 30 min each at RT to reduce non-specific antibody binding, followed by incubation with anti-neurofilament (NF) 200 mouse monoclonal antibody (clone NR52, Sigma; 1:500 in blocking solution) at 4°C overnight. After three washes in PBS (pH 7.4), the tissues were incubated with biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories Japan, Tokyo, Japan) for 1 h at RT. Following three washes in PBS (pH 7.4), the tissues were then reacted with ABC solution (Vectastain Elite kit; Vector Laboratories Japan) for 30 min at RT, according to the manufacturer's instructions. After further washing steps in PBS (pH 7.4), immunoreactivity was visualized by using the diaminobenzidine (Vector Laboratories Japan) reaction. After additional washes in distilled water, the cells were dehydrated through graded ethanol and xylene and then mounted on glass slides. The primary antibody was omitted from the procedure as a negative control; this gave no labeling corresponding to the immunostaining of the primary antibody.

Image presentation

Photomicrographic images of the immunostained SGNs were taken on a Nikon E800 microscope (Nikon, Tokyo, Japan) under brightfield illumination and phase-contrast optics with a digital microscope camera (AxioCam, Carl Zeiss Japan, Tokyo, Japan). Digital images were edited with Adobe Photoshop CS1 software (Adobe Systems Incorporated, Tokyo, Japan). The images were not modified except for minor adjustments of size, orientation, brightness, contrast and conversion to grayscale consistent with analysis.

Data analysis

We used the following indices for evaluation of the effects of neurotrophic factors on SGNs: the number of surviving neurons in each culture, the number of surviving SGNs relative to the initial seed number, the length of the longest neurite extending from each SGN and neuronal morphology. Data were collected from at least four independent experiments with at least duplicate wells for each of the experimental conditions. Quantitative analyses described below were performed by independent observers in a blinded manner.

To evaluate SGN survival, we counted all neurons present on each coverslip. Every cell that was labeled with the anti-NF200 antibody and also had a visible nucleus was counted as a surviving neuron, without regard to morphology or the number of processes.

To evaluate the length of the longest neurite on SGNs, neurons were sampled from the center field of view (4× objective) within each coverslip. Two additional sampling fields adjacent to the center field were employed in P20 cultures, because of the small number of SGNs in each field. The neurons within each 4× microscopic field were individually photographed and every SGN inside each field was included for analysis. All processes that emanated from the cell body of the SGNs were traced and their lengths were measured by using image analysis software (Microanalyzer, Polaroid Japan). The length of the longest neurite from each SGN was defined as the longest neurite length (LNL). The length of the neurite in SGNs without a neurite was listed as 0. When measuring neurite length, only those neurons whose processes could be clearly traced were included. This might have biased our results toward cells with shorter neurites, as some neurites exited the microscopic field. A total of 1247, 1355 and 953 neurons were analyzed from P0, P5 and P20 cultures, respectively.

Neuronal morphology was assessed for the same SGNs evaluated for the neurite extension. Each neuron analyzed was categorized into monopolar, bipolar, multipolar, or no processes. Neurons extending only neurite(s) shorter than the diameter of their cell body were categorized as having no processes. The percentage of the various morphologies was calculated from the total number of neurons analyzed.

Statistical analysis

Results were presented as the mean ± SE of samples in each experimental group. Statistical analyses for the number of surviving neurons and LNL were performed by using one-way analysis of variance (ANOVA) followed by Dunnet's post hoc test with GraphPad Prism 5 (GraphPad Software, La Jolla, Calif., USA) to compare each neurotrophin subgroup with the untreated control group. For the statistical analysis of the effect of neurotrophic factors on neuronal morphology, neurons were divided into two categories, i.e., SGN without or one neurite (referred to as the 0-1 neurite group) and SGN with two or more neurites (referred to as the ≥2 neurite group) and a chi-square test was used. Differences associated with P values of <0.05 were considered as statistically significant.

Results

General appearance of cultures

Immunostaining of the cultured cells at 4 h after plating revealed that the mean seed number of SGNs per well was 2387±88 in the P0 group, 889±65 in the P5 group and 164±18 in the P20 group.

Figure 1a illustrates an example of cultured SGNs after 12 h in primary growth medium containing serum and a further 72 h in serum-free maintenance medium. The surviving SGNs were identified as NF200-positive cells. As described in previous studies of murine dissociated SGN cultures (Vieira et al. 2007; Whitlon et al. 2007), cultured neurons either had no neurite (Fig. 1b) or showed monopolar (with one neurite emanating from the cell body; Fig. 1c), bipolar (with two neurites emanating from the cell body; Fig. 1d), or multipolar (with three or more neurites emanating from the cell body; Fig. 1e) morphologies.

We also examined the cultures under phase-contrast optics to evaluate the appearance of SGNs and non-neuronal cells (possibly consisting in glial cells and fibroblasts that were unlabeled). As shown in Fig. 2, non-neuronal cells in each culture exhibited two morphologies, i.e., flat-shaped cells with large nuclei and spindle-shaped cells with small nuclei and processes that extended from the cell body. This difference was most readily observed in P20 cultures (Fig. 2c). The density of non-neuronal cells decreased with increasing age of the donor animals; in P0 and P5 cultures, the non-neuronal cells formed a confluent layer on the glass surface (Fig. 2a, b), whereas the glass surface was partially free of non-neural cells in P20 cultures (Fig. 2c).