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Research Report

Immunolocalization of orphanin FQ in rat cochlea

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ABSTRACT

Orphanin FQ/nociceptin (OFQ/N) and its receptor (ORL-1) have been proposed to play a role in the regulation of hearing. In this study, we investigate the localization of OFQ/N-like immunoreactivity in the mammalian cochlea. Sprague–Dawley rat temporal bones were harvested and decalcified. The organ of Corti was microdissected, and indirect immunohistochemistry was performed using a rabbit polyclonal antibody raised against OFQ/N. Immunoreactivity was seen in the tunnel crossing fibers and the large boutons terminating onto outer hair cells, and in the fibers terminating onto the afferents to the inner hair cells. The findings are consistent with OFQ/N expression in lateral and medial olivocochlear efferents.

1. Introduction

Opioid peptides are a family of more than 20 endogenous neuromodulators derived from three precursor proteins, proopiomelanocortin, proenkephalin A and prodynorphin (Evans et al., 1988). Proopiomelanocortin generates the opioid peptide beta-endorphin. Met-enkephalin, leu-enkephalin, met-enkephalin RGL and met-enkephalin RF are major opioid peptides derived from proenkephalin A. Prodynorphin produces several dynorphins and neoendorphins.

Three types of opioid receptors have been identified at the molecular level: mu, delta and kappa opioid receptors (Kieffer, 1995). Based on homology to the opioid receptors, an additional receptor was cloned, the opioid receptor-like receptor (ORL-1) (Bunzow et al., 1994; Fukuda et al., 1994). All members of the opioid receptor family are 7-transmembrane G-protein coupled receptors with highly shared sequence identity. The 17-amino acid peptide, orphanin FQ/nociceptin (OFQ/N) which is derived from a larger precursor, prepro-OFQ/N (Reinscheid et al., 1998), was identified as the endogenous ligand of ORL-1 (Meunier et al., 1995; Reinscheid et al., 1995). ORL-1 binds selectively with OFQ/N with no appreciable affinity for the other opioid peptides (Dooley and Houghten, 1996; Reinscheid et al., 1996). It hyperpolarizes neurons by opening potassium channels (Hawes et al., 2000). Like other opioid agonists, OFQ/N inhibits adenylate cyclase and calcium channels. When injected intracerebro-ventrically or into specific brain regions, OFQ/N has been shown to influence neuroendocrine response (Devine et al., 2001), lordosis (Sinchak et al., 1997), feeding (Stratford et al., 1997), body

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temperature (Chen et al., 2001), locomotion (Florin et al., 1996), spatial learning (Sandin et al., 1997), and nociception (Heinricher et al., 1997; Dawson-Basoa and Gintzler, 1997; Mogil et al., 1996; Stanfa et al., 1996).

OFQ/N and its receptor, ORL-1, are widely expressed throughout the central nervous system. Based upon a number of criteria (including agonist-stimulated GTPγS binding, autoradiography, and mRNA levels), the abundance of this receptor in the brain is greater than that of any of the classical opioid receptors (Florin et al., 2000; Leon-Olea et al., 2001; Neal et al., 1999; Sim and Childers, 1997). The OFQ/N opioid system has a complex and widespread distribution in the perioesophageal ganglion of the snail, which is indicative that the OFQ/N opioid system had evolved early during the phylogenetic evolution (Leon-Olea et al., 2001). Using in situ hybridization and immunohistochemistry, Neal et al. demonstrated OFQ/N peptide and mRNA in almost all areas of the central nervous system from forebrain to spinal cord in the rat (Neal et al., 1999). [^{3}H] Labeled OFQ/N was used to localize ORL-1 and showed wide distribution throughout the central nervous system (Florin et al., 2000). OFQ/N activated G-proteins in the cortex, hippocampus and hypothalamus and the pattern of G-protein activation was distinct from that induced by the other opioid peptides (Sim and Childers, 1997).

Opioid peptides and opioid receptors are believed to play a role in the auditory neural innervation. There are prior immunohistochemical and biochemical studies demonstrating that the auditory olivocochlear efferents contain opioid peptides and that there are biochemically active mu, delta, and kappa opioid receptors in the mammalian cochlea (Altschuler and Fex, 1986; Altschuler et al., 1984, 1985; Eybalin and Pujol, 1984; Eybalin et al., 1987; Hoffman et al., 1985; Jongkamonwiwat et al., 2003; Ryan et al., 1991; Sahley and Nodar, 1994; Sahley et al., 1996). However, there are no prior studies on OFQ/N expression in the mammalian auditory periphery. There are two groups of olivocochlear efferents (Warr, 1992). The lateral olivocochlear (LOC) neurons have their cell bodies in the lateral regions of the superior olivary complex. These thin, unmyelinated LOC efferents primarily project to the ipsilateral cochlea and make axo-dendritic synapses with the radial afferents of the inner hair cells. However, they do not synapse directly onto the inner hair cells. The cell bodies of the medial olivocochlear (MOC) neurons lie in the medial portion of the superior olivary complex. These thick, myelinated MOC efferents project bilaterally, traversing the tunnel of Corti to synapse directly onto outer hair cells. Physiological studies indicate that the medial olivocochlear efferents contacting the outer hair cells adjust cochlear sensitivity through contractile responses of the outer hair cells (Dallos et al., 1997). Multiple neurochemicals have been identified in the olivocochlear efferents including considerable evidence for acetylcholine as the transmitter of the olivocochlear efferent system, and for γ-aminobutyric acid (GABA) efferent neurotransmission (for review see (Altschuler and Fex, 1986)). Researchers have proposed that OFQ/N plays a role in hearing regulation (Nishi et al., 1997). In this study, we use immunohistochemistry to determine the localization of OFQ/N expression in the rat auditory periphery.

2. Results

2.1. Controls

The cerebral neocortex of the animals served as the positive control (Figs. 1A and B). OFQ/N immunoreactivity was seen in multiple layers of the rat neocortex. The findings are consistent with prepro-OFQ/N in situ hybridization studies in mouse (Houtani et al., 2000) and rat (Neal et al., 1999). Immunohistochemistry using the commercially available OFQ/N antibody (Ab-cam) demonstrated similar distribution in the rat cochlea (not shown). Adsorption controls: the OFQ/N antibody was preincubated for 2 h at 37 °C with an excess of the purified peptide to which the antibody was raised. This peptide: antibody mixture was then diluted in blocking solution in replacement of the primary antibody step. Cerebral neocortex or cochlear tissue sections were processed for immunohistochemistry as described above. No immunoreactivity was detected in the negative controls (Figs. 1C and D respectively). Specificity for the antibody was tested by the use of various peptides for adsorption in immunohistochemistry including pro-enkephalin, leu-enkephalin, dynorphin, dynorphin-A, and endorphin. The immunoreaction in the rat cerebral cortex when pre-adsorbed with any of these peptides was unchanged

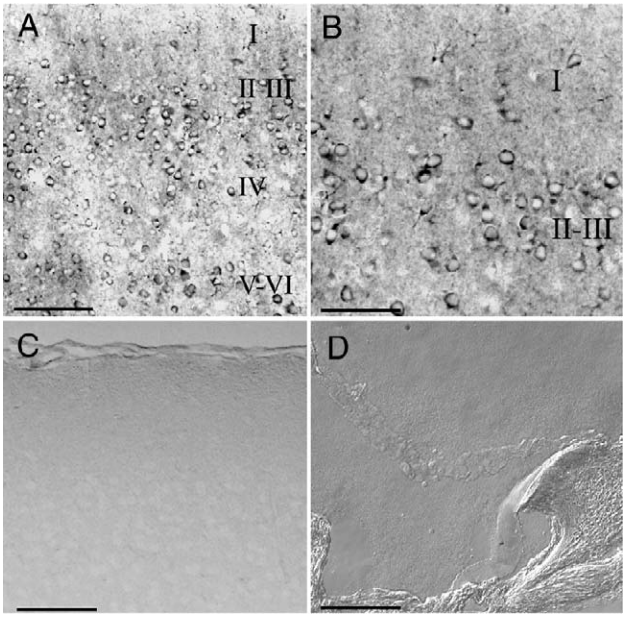

Fig. 1 – Positive control: Immunohistochemical staining of rat cerebral neocortex using OFQ/N antibody. Panel A is 10× magnification and Panel B is 40× magnification. There was moderate staining in neurons from layers IV–VI (A) and from layers II–III (B) neocortical layers. The staining pattern is consistent with in situ hybridization studies of prepro-OFQ mRNA expression in mouse (Houtani et al., 2000) and rat (Neal et al., 1999). Preadsorption negative control: no immunohistochemical staining was seen in rat neocortex (Figure C. 40× magnification) or the organ of Corti (Figure D. 40× magnification) when using OFQ/N antibody preadsorbed with OFQ/N peptide (Magnification bar in panels A and C=300 μm, in panel B=150 μm, and in panel D=100 μm).

from the positive control (not shown). Specificity for the OFQ/N antibody was proven by immunoreaction on the cerebral cortex and hippocampus of OFQ–/– (homozygous knockout mouse), and no immunoreaction was noted (not shown).

2.2. *Immunolocalization of OFQ/N expression*

There were OFQ/N-immunoreactive signal within the tunnel crossing fibers noted in whole mount cochlea preparations (Figs. 2A, B). Large terminals making contact at the basal outer hair cell were OFQ/N-immunoreactive (Fig. 2B). Using indirect immunofluorescence OFQ/N immunoreactive terminals were seen underneath inner hair cells (Fig. 2C). These findings are consistent with OFQ/N-immunoreactivity in the medial olivocochlear efferent terminals that synapse directly onto the outer hair cells. In thin serial cross-sections, OFQ/N-immunoreactive fibers were noted in the thin fibers and terminals underneath the inner hair cell; the terminals did not contact the inner hair cell directly (Figs. 3A–E, Fig. 4). These findings are consistent with OFQ/N-immunoreactivity in the lateral olivocochlear efferents which synapse onto the inner hair cell afferents. The tunnel crossing fibers and the large terminals beneath the three rows of outer hair cells were OFQ/N-immunoreactive. OFQ/N-immunoreactive fibers (Fig. 5A) terminated in prominent immunoreactive large boutons beneath the outer hair cells (Fig. 5B). The outer hair cell, inner hair cell and supporting cells in the organ of Corti were non-immunoreactive.

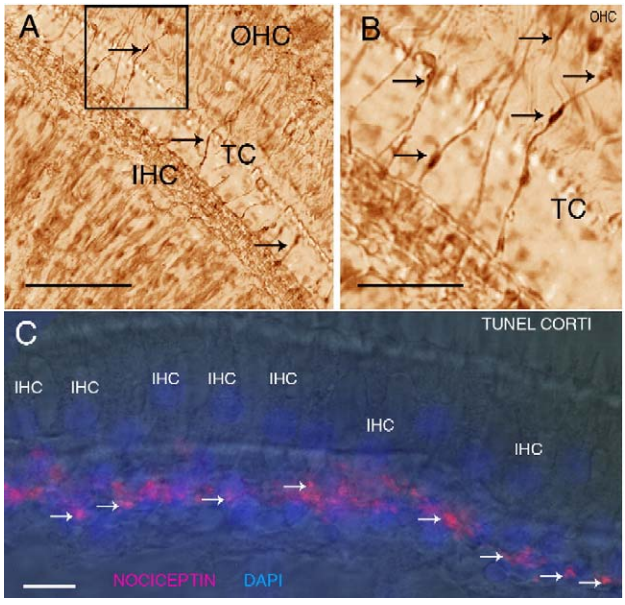

Fig. 2 – OFQ/N immunoreactivity in a whole mount preparation of the rat organ of Corti. Arrows point to immunoreactive punctae in the tunnel crossing efferent fibers. (A) 60× *magnification*. (B) is high (100×) magnification of the boxed area within panel A. (C) Immunoreactive terminals were visualized using immunofluorescence (in red color, and arrows pointing the immunoreactive terminals, in blue is DAPI to visualize cell nuclei, bright field image was superimposed to appreciate the different structures). OFQ/N immunoreactivity was also seen in large terminals at the base of outer hair cells (arrows at upper right). (IHC, inner hair cells; OHC, outer hair cells. PC Pillar cells; TC, tunnel of Corti; Magnification bar in panel A=75 μm, in panel B=25 μm. In panel C=8 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Discussion

There is strong evidence for opioid peptides and opioid receptors in the mammalian auditory periphery. Enkephalins and dynorphin-like immunoreactivities localized to the lateral olivocochlear efferent system in the guinea pig and the cat (Altschuler and Fex, 1986; Altschuler et al., 1984, 1985; Eybalin and Pujol, 1984; Hoffman et al., 1985) and enkephalin mRNA expression localized to half of the medial olivocochlear efferents in the gerbil (Ryan et al., 1991). Physiological studies demonstrated that (–) pentazocine, a ligand that binds specifically to kappa opioid receptor, infused intravenously into the chinchilla was associated with decreased sound thresholds (Sahley and Nodar, 1994), an effect reversed by naloxone (Sahley et al., 1996). There is also evidence for biochemically active mu and delta opioid receptors in the cochlea. Eybalin et al. (1987) demonstrated inhibition of adenylate cyclase in guinea pig cochlear homogenate in vitro by using a mu specific agonist and a delta specific agonist, an effect which was reversed by opioid receptor antagonist. Immunohistochemistry of the rat auditory periphery localized delta opioid receptors in the inner hair cells, outer hair cells, and bipolar cells of the spiral ganglion and interdental cells of the limbus; mu opioid receptor staining was seen in the supporting cells, the area beneath the outer hair cells and bipolar spiral ganglion cells and kappa opioid receptor staining was seen in the inner hair cells, the outer hair cells and the bipolar cells (Jongkamonwiwat et al., 2003).

It has been proposed that the ORL-1 receptor is involved in hearing regulation because homozygous ORL-1 knockout mice demonstrate dysregulation of hearing (Nishi et al., 1997). Both homozygous and heterozygous ORL-1 knockouts mice have normal temporal bone morphology and normal baseline auditory thresholds. However, when exposed to loud noise, the auditory thresholds for homozygotic knockouts increased as opposed to those of the wild type and heterozygotes, which remained at baseline. The auditory thresholds of the homozygotes normalized after 3 days (Nishi et al., 1997).

Investigations in the mammalian auditory brainstem have demonstrated strong evidence for OFQ/N expression (Houtani et al., 2000; Kakimoto et al., 2001; Neal et al., 1999). Using immunohistochemistry and in situ hybridization, OFQ/N peptide and mRNA was expressed in the nucleus of the trapezoid body, the superior paraolivary nucleus, medioventral periolivary nuclei, and scattered cells in the dorsal, lateroventral and superior periolivary nuclei and in the lateral superior olive of the rat (Neal et al., 1999). The nucleus of the trapezoid body contains the medial olivocochlear neurons and the lateral superior olive contains the majority of the lateral olivocochlear neurons in the rat (Warr, 1992). A study using X-gal immunohistochemistry in the mouse reported the localization of the ORL-1 receptor in the central auditory

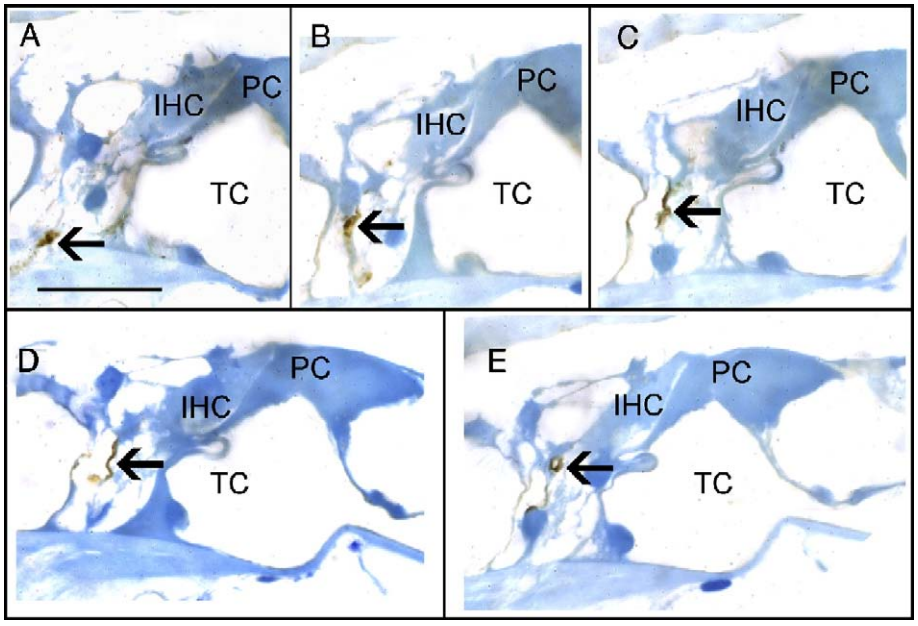

Fig. 3 – OFQ/N immunoreactivity in the fibers and terminals underneath the inner hair cells. Arrows point to immunoreactive fibers which terminate beneath the inner hair cells. The fibers do not contact the inner hair cells, a pattern consistent with lateral olivocochlear efferent fibers. The inner hair cells and pillar cells are non-immunoreactive. (A–E) are serial thin tissue sections, counterstained with toluidine blue. (IHC, inner hair cells; PC, pillar cells; TC, tunnel of Corti; Magnification bar in panels A–E = 15 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pathways of the dorsal and ventral division of the lateral lemniscus, the superior periolivary region, the dorsal cochlear nucleus and ventral cochlear nucleus (Houtani et al., 2000). Kakimoto et al. reported prepro-OFQ/N mRNA expression in the dorsal and ventral nuclei of the lateral lemniscus, the rostral periolivary region, the superior paraolivary nucleus, the cortex of the inferior colliculus, the dorsal periolivary region with especially dense expression in the lateroventral and medioventral periolivary nuclei (Kakimoto et al., 2001). The medioventral and lateroventral periolivary nuclei are the major sites of olivocochlear neurons in mouse (Warr, 1992). Notably, the areas of the brainstem which contain the neuronal cell bodies of the lateral and medial olivocochlear efferents express OFQ/N peptide and mRNA, and the precursor pre-OFQ mRNA. Thus, it would be predicted that OFQ/N peptide is expressed in the medial and lateral olivocochlear efferents in the cochlear endorgan.

This study provides the first immunohistochemical study of OFQ/N localization in the mammalian auditory periphery, specifically localizing to both the medial and lateral olivocochlear

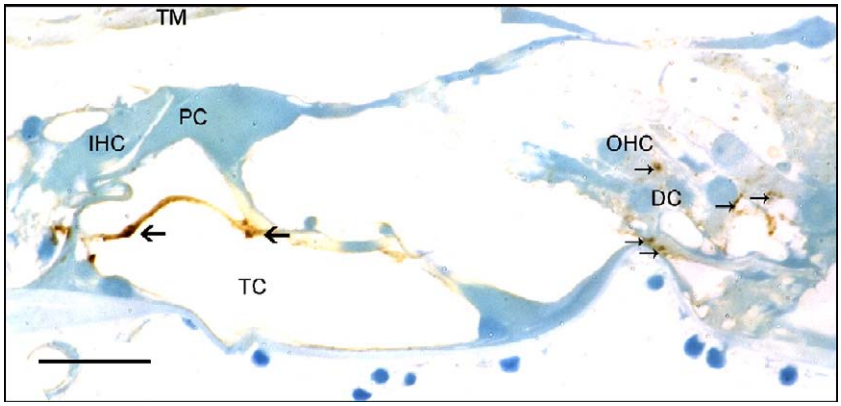

Fig. 4 – OFQ/N immunoreactivity in tunnel crossing fibers. Immunoreactive tunnel crossing fiber staining can be seen (bold arrows). At the right side of the photomicrograph, there was immunohistochemical reaction in large boutons contacting the outer hair cells (small arrows). The inner (IHC) and outer hair cells (OHC), Deiter's cells (DC) and pillar cells (PC) are non-immunoreactive. Thin tissue section, counterstained with toluidine blue. 60× magnification. (TC, tunnel of Corti; Magnification bar = 15 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

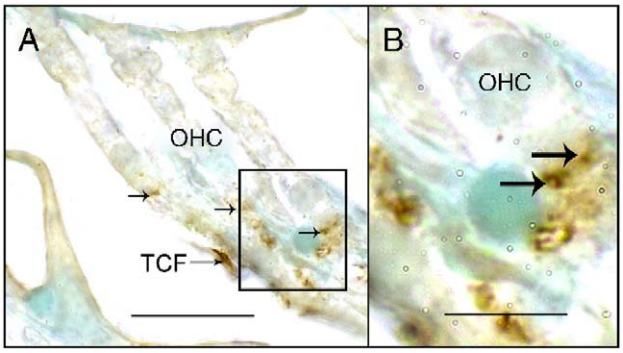

Fig. 5 – OFQ/N immunoreactivity in large terminals underneath outer hair cells and tunnel crossing fibers. (A) Arrows point to the tunnel crossing fibers (TCF) that were strongly immunoreactive. (B) The arrows point to bouton staining under the outer hair cells. Thin tissue section, counterstained with toluidine blue. (OHC, outer hair cells; Magnification bar in panel A = 15 μm, and in panel B = 5 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

efferents. In the present study, OFQ immunostaining pattern is consistent with lateral olivocochlear efferent expression of OFQ/N, with immunoreactive fibers terminating in the area beneath the inner hair cells, presumably on afferents. OFQ/N immunoreactivity was also present in the tunnel crossing fibers and in the large boutons contacting the outer hair cells, a staining pattern that is consistent with medial olivocochlear efferent expression of OFQ/N. Our results are in agreement with previous studies in which OFQ/N and the precursor prepro-OFQ/N localize to the medial and lateral regions of the superior olivary complex (Houtani et al., 2000; Kakimoto et al., 2001; Neal et al., 1999), which form the origin of the medial and lateral olivocochlear efferent pathways, respectively.

The localization of OFQ/N in the olivocochlear efferents provides further evidence that OFQ/N plays a role in cochlear efferent neurotransmission or neuromodulation. At other neurological sites, OFQ/N apparently acts on presynaptic release sites to inhibit the release of glutamate in the amygdala (Meis and Pape, 2001). In the suprachiasmatic nucleus, the baseline calcium ion level in the presynaptic terminals was consistently reduced by OFQ/N interaction with N- and P/Q-type voltage gated calcium channels, blocked by the specific calcium channel antagonists omega-conotoxin GVIA (N-type) and omega-agatoxin TK (P/Q-type) (Gompf et al., 2005). Glutamate is believed to be the primary afferent neurotransmitter released from the inner hair cells (Sewell, 1996). Lopez et al. demonstrated immunohistochemical evidence for P/Q-type (alpha 1A) and L-type calcium channels (alpha 1D) in the inner and outer hair cells of the chinchilla (Lopez et al., 2003). Therefore, OFQ/N released from the lateral olivocochlear efferents may possibly modulate auditory neurotransmission by the inhibition of cochlear hair cell glutamate release, if OFQ/N modulates via volume transmission, or by the modulation of P/Q calcium channels in the inner and outer hair cells.

4. Experimental procedures

4.1. *Animal welfare act compliance*

Ten young rats (1–2 month of age) were used in the present study. Animals were handled and cared for in accordance with the Animal Welfare Act and in strict compliance with the National Institutes of Health Guidelines. The Chancellor's Animal Subject Protection Committee at the University of California, Los Angeles, approved the research protocols for the use of animal subjects in this study.

4.2. *Characterization and source of antibodies*

OFQ/N rabbit polyclonal antibody (kindly donated by Christopher J. Evans, UCLA) was generated in rabbit by using the OFQ/N peptide as the immunogen. This antibody reacts with rat and mice frontal cortex and cochlea sections. Additionally, Ab-cam rabbit polyclonal antibody against OFQ/N was used to collaborate the findings (Ab-cam Laboratories, catalog ab6174). The immunogen is the OFQ/N peptide with a cysteine added at the N terminal for conjugation to diphtheria toxoid. The peptide reacts with guinea pig and rat.

4.3. *Tissue processing*

Rats were over anesthetized with halothane. Upon cervical dislocation, animals were decapitated. The temporal bones containing the inner ear were removed from the skull. Ears were immersed in 4% paraformaldehyde (dissolved in 0.1 M sodium phosphate buffer, pH 7.2) for 24 h, and decalcified by immersion in 3% EDTA buffered phosphate solution for 5 days. The inner ear was processed as follows. In order to make whole-mount sections, the bone surrounding the cochlea from the decalcified temporal bone was carefully removed. The whole microdissected cochlea was cut into three pieces (apical, medial and basal), and immersed in phosphate-buffered saline solution to perform immunohistochemical staining. In order to make mid-modiolar cryostat sections, the auditory bullae were further microdissected and immersed in 30% sucrose for 3 days. The whole cochlea was immersed in OCT compound (Polysciences, Inc.), and properly oriented, and frozen inside a cryostat (model Micron HN505E) to obtain mid-modiolar sections. Fourteen-micron thin serial sections were made. Sections were mounted on Superfrost plus slides, and stored at −80 °C until their use.

4.4. *Immunohistochemistry for controls*

Antibody specificity was tested by the use of various peptides for adsorption in immunohistochemistry. In brief, the OFQ/N antibody was adsorbed with enkephalin (Santa Cruz, CA), dynorphin and endorphin (Sigma SLM) peptides. The mixture was applied to rat or mice cerebral cortex and compared with the positive control immunohistochemistry of rat cerebral cortex. Immunohistochemistry using the same steps as described below was conducted using the OFQ/N antibody on homozygous OFQ/N−/− mouse. In addition, the commercially available OFQ/N rabbit polyclonal antibody (Ab-cam)

(1:1000) was applied to the cerebral cortex of rat and mouse for further confirmation of results.

4.5. *Immunohistochemistry for light microscopy*

The tissue specimens or cochlea segments were incubated for 10 min in 0.3% hydrogen peroxide (Fisher Scientific) diluted in 50% methanol in PBS. Tissue specimens or cochlea segments were rinsed in PBS (15 min × 3) between each subsequent step. The tissue specimens or cochlea segments were incubated at room temperature for 1 h with a blocking solution containing 1% normal goat serum, 1% bovine serum albumin in 0.2% Tween in PBS. After the blocking solution was removed, primary rabbit polyclonal antibody against OFQ/N (kindly supplied by Dr. Christopher Evans, UCLA) diluted 1:500 in blocking solution was applied to the tissue. Tissue specimens were incubated in a humid chamber at 4–6 °C for 48 h. Whole cochlea tissue was placed in a rotator shaker and incubated in the cold room at 4–6 °C for 48 h. Biotinylated goat anti-rabbit secondary antibody (Elite kit, Vectors Labs) diluted 1:1000 in PBS with 1% NGS and 1% BSA was applied and cochlea was incubated for 1 h at room temperature. Avidin–biotin–peroxidase solution (Elite kit, Vectors Labs) was applied for 1 h at room temperature. The immunohistochemical reaction was visualized by incubating the tissue sections with a diaminobenzidine solution (DAB kit, Vector Labs) for 3–5 min at room temperature. The reaction was halted with distilled water (15 min × 3). At the end of the incubation, sections were mounted with Vectashield solution (Vector Labs). Tissue specimens were viewed and imaged in a Nikon Eclipse E800 microscope equipped with RTSlider spot digital camera and Image Pro Plus™ software.

4.6. *Histological processing of immunoreacted tissue*

Immunoreacted specimens used for whole mount analysis were post-fixed 0.1% osmium tetroxide solution in PBS for 1 h. The tissue was dehydrated with ascending alcohols, propylene oxide, infiltrated and embedded in EPON-810. Five-micron thick sections were obtained using a Micron Ultramicrotome HM355-S. Sections were counterstained with 0.1% toluidine blue solution and cover-slipped with Permount mounting media (Fisher).

4.7. *Immunofluorescence*

For immunofluorescence staining cochlear tissue was incubated at room temperature for 1 hr with a blocking solution containing 1% BSA grade V (Sigma), 0.1% Triton X-100 (Sigma) in PBS. Next the solution was removed and the primary polyclonal antibodies against OFQ/N (Ab CAM 1:500) were incubated overnight at 4 °C in a 1.5 ml centrifuge tube. The secondary antibodies against rabbit labeled with 594 (1:1000, for red color, Molecular probes) were applied and incubated for 1 h at room temperature in the dark. At the end of the incubation, the tissue was washed with PBS and mounted with Vectashield solution containing DAPI (Vector Labs, Burlingame CA) to visualize all cell nuclei.

Light and fluorescent microscopic observation and documentation: whole mounts of the cochlea and tissue sections were viewed and imaged in a Nikon Eclipse E800 microscope equipped with RTSlider spot digital camera and Image Pro Plus™ software. Images were processed using the Adobe Photoshop software program run in a Macintosh iMAC computer.

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