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# Detection of extracellular signal-regulated kinase1/2 in the inner ear of guinea pigs

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**Abstract**

Growth factors, such as vascular endothelial growth factor (VEGF) and neurotrophins, recently identified in the inner ear of guinea pigs, exert their proliferative properties partly through activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK1/2). In order to demonstrate presence of ERK1/2 in the inner ear we performed immunohistochemical analysis using specific antibodies to inactive and activated ERK1/2 on paraffin-sections of temporal bones from guinea pigs ($n = 5$). In the cochlea clear immunoreactivity to inactive ERK1/2 was predominant in the spiral ligament, in the organ of Corti (intensive staining in supporting cells, faint staining in sensory cells) and limbus epithelium, while spiral ganglion cells and nerve fibres revealed weak staining. Activated ERK1/2 could be detected sparely in the spiral ligament exclusively. In the vestibule inactive ERK1/2 was located in the sensory epithelium, in nerve fibres and in vascular endothelium, while activated ERK1/2 could be detected in few nerve fibres and synaptic endings (buttons and calyces) on hair cells of the maculae and crests and in the endothelium of few blood vessels. These findings provide evidence that activated ERK1/2, as a general downstream signal of growth factors, may be contributed in the inner ear physiology. 

*Keywords*: Mitogen-activated protein kinase; Extracellular signal-regulated kinase1/2; Inner ear; Guinea pig

Diverse growth factors, such as vascular endothelial growth factor (VEGF) [4] and neurotrophins including their receptors [2,7,10], have been identified in the guinea pig inner ear recently. The mitogenic effects of growth factors are partly mediated through activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) [9,15,16]. Up to now, reports concerning MAPK/ERK1/2 as a part of a downstream signalling pathway of VEGF or neurotrophins in the inner ear are not available yet.

Three-month-old female guinea pigs ($n = 5$) were anesthesized with pentobarbital and sacrificed via cervical dislocation. Fixation was obtained by cardiac perfusion with fixative (4% paraformaldehyde, buffered at pH 7.4) after flushing out the blood cells with phosphate-buffered saline (PBS) (0.1 M at pH 7.4). Both temporal bones of each animal were removed, the bulla opened and immersed in the same fixative for 12 h. Decalcification was performed with ethylenediaminetetra-acetic acid (EDTA) solution 10%, buffered with Tris at pH 7.0 over 7 days. The tissue was embedded in paraffin for immunohistochemical analysis and sectioned with a microtome (Microm HM 360) in slices of 8 μm thickness. After removal of paraffin with ethanol and xylene, the sections were incubated over night at 4°C separately with a commercial antibody (Ab) to activated (diphosphorylated) ERK1/2 (Sigma, monoclonal anti-MAP kinase, clone MAPK-YT mouse ascites fluid M8159, diluted at 1:400). For detection of inactive ERK1/2 a specific Ab bought from Upstate Biotechnology (rabbit immunoaffinity purified IgG #06–182, lot #18597, diluted at 1:400) was used. Negative controls were obtained by omitting the primary antibodies. After rinsing with Triton-PBS 0.1% and normal-goat-serum, a biotinylated goat anti-rabbit, or anti-mouse Ab respectively, (1:400/Dako) was used for accentuation. Processing was performed by means of streptavidin-biotinylated horseradish-peroxidase complex (1:100/Amersham) using nickel enhanced DAB

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staining, with subsequent analysis and photography of the sections under a Zeiss Axiophot light-microscope.

ERK1/2 (inactive)-immunostaining: Inactive ERK1/2 was found widely distributed in vestibulocochlear structures and could be found all specimens. Strongest immunostaining could be detected in the spiral ligament, in the limbus, in

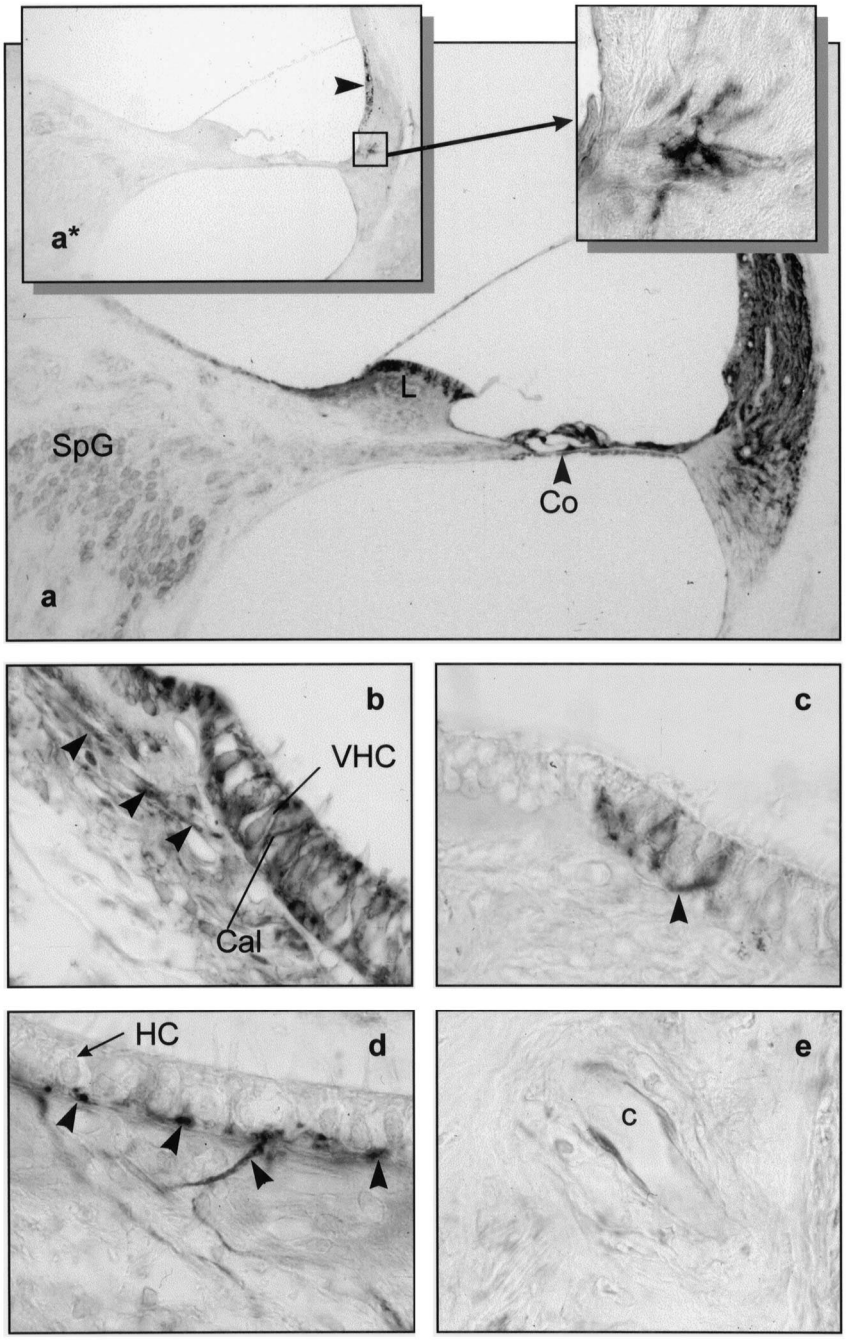


Fig. 1. (a) Anti-(inactive)ERK1/2-immunostaining, 8 μm paraffin section, 10×: strong immunoreactivity can be noted in the epithelium of the limbus (L), in supporting cells of the organ of Corti (Co) and in the ligamentum spirale. Weak labelling is found in the sensory cells (inner and outer hair cells) and perikarya of the spiral ganglion (SpG). (a*) Activated ERK1/2 in the cochlea was exclusively found sparely in the ligamentum spirale (square) possibly belonging to endothelial cells. The dark spots in the stria vascularis (arrowhead) is caused by natural accumulation of pigment and can be found also in unprocessed sections. (b) Anti-(inactive)ERK1/2-immunostainíng, 100×: immuno-labelling is present in nerve fibres (arrowheads), wall of blood vessels and in nerve endings. Within the sensory epithelium clear staining is displayed in supporting cells, calyces (Cal) and nerve endings are clear labelled, vestibular hair cells (VHC) are displaying faint staining. (c) Anti-(activated)ERK1/2, 100×, the corresponding region to (b), the basis of a crest is shown. Clear immunoreactivity can be stated in the synaptic calyces (arrowhead points at corresponding nerve fibre) of some type I hair cells. (d,e) Anti-(activated)ERK1/2, 100×, positive staining is occasionally found in nerve fibres and synaptic buttons (arrowheads) on hair cells of the maculae (d) and in some endothelial cells of subepithelial vessels in the vestibule (c-capillary) (e).

sensory epithelia (Organ of Corti, maculae and cupulae), in nerve fibres and in endothelial cells (Fig. 1a,b). Only faint staining was found in vestibular and spiral ganglion cells. The stria vascularis showed beside a natural accumulation of pigment no specific immuno-labelling. Within the organ of Corti, inner and outer hair cells showed faint staining, while supporting cells as cells of Deiter, inner and outer pillar cells revealed clear staining (Fig. 1a). In the maculae and cristae strongest labelling became evident in supporting cells and in calyces, while vestibular hair cells showed weaker staining (Fig. 1b). Furthermore, endothelium of blood vessels of the whole temporal bone including the modiolus, lateral wall and the vestibule revealed clear staining ERK1/2 (activated)-immunostaining. In the cochlea activated ERK1/2 was found exclusively in the spiral ligaments of basal and middle turns (three of five cochleae), possibly belonging to vascular epithelium (Fig. 1a*).

In the vestibule sensory epithelium activated ERK1/2 could be found regularly in a few calyces around vestibular hair cells (Fig. 1c) and in few nerve fibres, including their synaptic endings (buttons) (Fig. 1d). Furthermore, vascular endothelium was found labelled occasionally (Fig. 1e). Perikarya remained unstained.

This study provides immunohistochemical evidence that inactivated ERK1/2 is expressed in almost all important structures of the adult guinea pig inner ear. Under normal conditions only a few percent of ERK1/2 is present in the activated form, suggesting a possible role for vestibular physiology. ERK1/2 is an essential component of the neurotrophin/trk-receptor pathway [9] and an alternative downstream signal pathway for VEGF [16], which has been described to be involved in the nitric oxide/cyclic guanosine monophosphate (cGMP) pathway [1,5,11]. Presence of neurotrophins and trk [2,7,10], as well as VEGF and VEGF-receptors [4] in the inner ear has been demonstrated recently. The detection of ERK1/2 rises evidence for a general downstream signal pathway for neurotrophins and VEGF, which may mediate neurotrophic and neuroprotective activity, stimulation of axonal outgrowth and enhancement of cell survival and proliferation in the peripheral nervous system [12,13] as well as regulation of ion channels [14] in the inner ear. Furthermore, it has been shown recently that also nitric oxide (NO) and cGMP – meanwhile all compounds of the NO/cGMP pathway have been detected in the inner ear [3,6] – are contributed to VEGF-dependent ERK1/2 activation [8].

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