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Identification of auditory neurons by retrograde labelling for patch-clamp recordings in a mixed culture of chick brainstem

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Abstract

We present a method to identify specific sub-populations of auditory neurons in a mixed primary cell culture of the chicken brainstem, allowing the study of individual neurons with a known identity *in vitro*. To label specific afferent cell types, we injected retrograde tracers (dextrans coupled to fluorescent dyes) into either the mid-line or the superior olivary nuclei (SON) of the isolated chicken brainstem *in vitro*. Mid-line injections resulted in stable labelling of neurons of the nucleus magnocellularis (NM), whereas injections into the SON retrogradely labelled neurons of the nucleus laminaris (NL). The fluorescent label survives the dissociation procedure and is detectable for at least 1 week *in vitro*. Only about 0.1% of all cells *in vitro* are pre-labelled. The auditory identity of the pre-labelled neurons was confirmed with calretinin immunocytochemistry and electrophysiological recordings, where the cells had typical firing patterns of auditory brainstem neurons. In the future, this method can be combined with single cell PCR to match nuclear origin, firing patterns and the expression of functional molecules *in vitro*.

Keywords: Bird; Avian brainstem; Dextran; Primary cell culture; Tracing *in vitro*

1. Introduction

Primary neural cultures are a useful and well-established method to answer questions in cellular neurobiology. However, primary neural cultures often contain a mixture of different cell types, while most researchers are interested in only a single cell type. Often, these cell types cannot be distinguished by morphological criteria *in vitro*, but only by immunocytochemistry or *in situ* hybridisation. Since fixation is needed for these techniques, electrophysiological recordings of identified neurons *in vitro* are impossible.

To overcome these restrictions primary culture systems of pre-labelled neurons were developed. The most elegant way to label a desired neuronal population is genetical engineering. Fluorescent proteins driven by an appropriate promoter can distinctively label populations of neurons (Furuta et al., 2004). Unfortunately, this method is not easily available for chicken. An alternative approach is to stain functional surface molecules. This can be done by incorporation of radioactive isotopes needed for the synthesis of surface molecules, e.g. $^{35}SO_4$ for sulphated glycolipids of high endothelial cells from rat lymph nodes (Ager, 1987). Another possibility is the fluorescent immunolabelling of surface proteins and a subsequent cell sorting (Abney et al., 1983) or an immunocoupling of iron particles followed by a magnetic separation (Moenig and Luksch, 2006).

A fourth method is the retrograde tracing of target neurons which is easy if axonal connections are well known. This method of labelling was extensively used to create primary cultures of retinal ganglion cells (Sarthy and Balkema, 1981; Ishida and Cohen, 1988; Hong and Thanos, 1996) but also for explant cultures of trigeminal sensory neurons (O'Connor and van der Kooy, 1988) and neurons of the major pelvic ganglia (Yoshimura et al., 1994). Different molecules are suitable for retrograde transport. True blue (Sarthy et al., 1983; O'Connor and van der Kooy, 1988; Yoshimura et al., 1994), horseradish-peroxidase (Ishida and Cohen, 1988) and biotin (Lapper and Bolam, 1991) have all been successfully employed to retrogradely label neurons. There are different methods to visualize these tracers. True blue is a fluorescent dye in itself. Horseradish-peroxidase is an enzyme that, upon presence of

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H_2O_2, converts di-amino-benzidine to a brown reaction product. Biotin is very versatile and can be visualized via avidine coupled to different dyes and enzymes. Other tracers that are extensively used are dextrans of different size (Rajakumar et al., 1993). For neuronal tracing dextran of a molecular weight of 3000 Da seem to be optimal (Fritzsch, 1993). Comparable to biotin, dextrans can be coupled to different enzymes and dyes.

Our group studies the auditory brainstem of chicken. In the chicken brainstem nucleus angularis (NA), nucleus magnocellularis (NM) and nucleus laminaris (NL) contain the second and third order neurons of the auditory pathway. The neuronal circuit composed of NM and NL neurons code for interaural timing differences (Overholt et al., 1992) and is highly specialised for faithful and fast transmission of excitation (Reyes et al., 1994, 1996). A typical marker of these neurons in birds is the calcium-binding protein calretinin (Parks et al., 1997; Kubke et al., 1999). The development of the auditory brainstem and the pattern of afferent and efferent connections of the NM and NL have been described in detail (Overholt et al., 1992; Hendricks et al., 2006). Neuronal projections of the NM cross the mid-line and target neurons of the NL from the ventral side. Ipsilateral projections of NM neurons target the NL neurons from the dorsal side. NL neurons and NA neurons but not NM neurons project to the superior olivary nucleus (SON) (Lachica et al., 1994). The SON projects to all three auditory nuclei. Therefore, injection of tracer into the SON will label the somata of NA and NL neurons while injection into the mid-line of the brainstem will label the somata of NM neurons.

While morphological features of this system seem to develop at least partially independent of sensory input (Parks et al., 1987), the development of physiological specialisations of NM and NL neurons is less well understood. Recently we published a study on the development of a mixed culture of chicken auditory brainstem neurons (Kuenzel et al., 2007). We could show that these neurons express calretinin and characteristic voltage-gated potassium channels Kv3.1b *in vitro*, and we could also identify different firing types by whole-cell patch recordings. Unfortunately, it was impossible to match these electrophysiological properties to specific cell types. To overcome these limitations, we developed a primary culture system of pre-labelled auditory brainstem neurons.

2. Material and methods

2.1. Animals

Fertilised eggs of White-Leghorn chicken obtained from a local poultry farm were incubated in a forced draft incubator for 10–11 days. After egg opening and prior to preparation, the stage of the embryo was confirmed according to Hamburger and Hamilton (1951). All preparations were performed under a lateral air-flow sterile workbench. Chicken embryos (E10-11) were decapitated and the head was rinsed in ice-cold Ringer solution. The frontal part of the head containing the forebrain was cut away with a scissor. The posterior part of the head was put on the dorsal side and the covering tissue was removed with two forceps. The posterior brain was carefully taken out of the skull and rinsed with cold Ringer.

2.2. Retrograde tracing

Injection cannulae were prepared from glass capillaries (1.2 mm × 0.68 mm, A-M Systems Inc., Everett, WA, USA) on a horizontal puller (DMZ Universal Puller, Zeitz-Instrumente, Augsburg, Germany). The tips were broken and the capillaries were filled with oil and mounted in a nanoliter-injector (WPI, Sarasota, FL, USA). Dextranamine (MW 3000) coupled with Alexa488, Alexa546 or rhodamine (Molecular Probes, Invitrogen GmbH, Karlsruhe, Germany) was dissolved in PBS and 10% dimethylsulphoxide to a final concentration of 10% and filled in the capillary.

After removal of the meninges 6–8 injections of 4.6 nl each were placed either from the dorsal side into the mid-line of the brainstem (Fig. 1A and C) or from the ventral side into the rostral part of the brainstem (Fig. 1B and C). After injection the brains were collected in oxygenated sterile Ringer's solution and *ex vivo* tracing took place for 4–5 h at room temperature in darkness.

2.3. Histology

After *ex vivo* tracing the brainstem was fixed by immersion in 4% paraformaldehyde in phosphate-buffer (PB) at 4 °C over night. Afterwards, it was cryo-protected in 30% sucrose in PB. The brainstem was cut with a cryostat (CM3050, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) into slices of 60 μm thickness. The sections were incubated in 4′,6-diamidine-2-phenylindol (DAPI) solution (1:50,000 in PB) for 5 min and mounted with Fluoroprep (BioMerieux).

2.4. Cell culture

After *ex vivo* tracing the brains were transferred into ice-cold sterile HBSS. Tissue chunks containing the NM and NL neurons were prepared from the traced brainstems. NM and NL were excised under a preparation microscope (Olympus, Hamburg, Germany) using pieces of razor blades (Fig. 1C, dotted lines). For a single culture preparation, tissue from 3 to 4 embryos was pooled. Tissue chunks were treated with trypsin/EDTA (Gibco/Invitrogen GmbH, Karlsruhe, Germany) for 14 min and gently triturated using 1 ml plastic pipet tips. Cells were spun down (5 min, 300 × *g*), briefly resuspended in DMEM:F12 (Gibco/Invitrogen) containing 10% fetal calf serum (FCS, Gibco/Invitrogen) to stop trypsin activity, and rinsed once with HBSS before being resuspended in the serum-free culture medium, DMEM:F12 containing 2% of the serum supplement B27 (Gibco/Invitrogen). All media contained 20 units/ml penicillin/streptomycin. Cells were seeded on glass coverslips coated with poly-D-lysine (0.1 mg/ml, at least 1 h at 37 °C) in a density of 500 live cells/mm^2. The cultures were kept in a humidified incubator at 37 °C and 5% CO_2 atmosphere.