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The mechanisms and possible sites of acetylcholine release during chick primary sensory neuron differentiation

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ABSTRACT

Aims: In this study, we evaluated the ability of differentiating embryonic chick DRG neurons to release and respond to acetylcholine (ACh). In particular, we investigated the neuronal soma and neurites as sites of ACh release, as well as the mechanism(s) underlying this release.
Main methods: ACh release from DRG explants in the Campenot chambers was measured by a chemiluminescent assay. Real-time PCR analysis was used to evaluate the expression of ChAT, VAChT, mediatophore and muscarinic receptor subtypes in DRGs at different developmental stages.
Key findings: We found that ACh is released both within the central and lateral compartments of the Campenot chambers, indicating that ACh might be released from both the neuronal soma and fibers. Moreover, we observed that the expression of the ChAT and mediatophore increases during sensory neuron differentiation and during the post-hatching period, whereas VAChT expression decreases throughout development. Lastly, the kinetics of the m2 and m3 transcripts appeared to change differentially compared to the m4 transcript during the same developmental period.
Significance: The data obtained demonstrate that the DRG sensory neurons are able to release ACh and to respond to ACh stimulation. ACh is released both by the soma and neurite compartments. The contribution of the mediatophore to ACh release appears to be more significant than that of VAChT, suggesting that the non-vesicular release of ACh might represent the preferential mechanism of ACh release in DRG neurons and possibly in *non-cholinergic* systems.

Introduction

It was proposed that, in addition to their classical role in synaptic transmission, neurotransmitters (NTs) have been proposed to play functional roles both during neurogenesis and during adult life (Buznikov et al., 1996; Biagioni et al., 2002; Karczmar, 2007; Bovetti et al., 2011; Young et al., 2011; Abreu-Villaça et al., 2011).

Cholinergic components have been identified in non-neuronal cells as part of autocrine or paracrine signaling systems (Loreti et al., 2006; Kawashima and Fujii, 2008). Thus, NTs appear to be "old" molecules that are present in many organisms and that participate in signaling mechanisms that control various cellular functions such as proliferation and migration. Their role in neurogenesis has been proposed on the basis of their early appearance during development, which extends well into the advanced stages of synaptogenesis (Bovetti et al., 2011; Young et al., 2011).

The role of acetylcholine (ACh) as a cofactor of neuronal differentiation has been investigated in mouse neuroblastoma cell lines and dorsal root ganglia (DRGs) (Biagioni et al., 2002; Salani et al., 2009). DRG neurons are not a single population; they display both morphological and biochemical differences in relation to their ability to receive diverse physico-chemical signals from all body structures, thus ensuring responses to peripheral stimuli and their integration (Salt and Hill, 1983). Despite their different properties, DRG neurons share the unique morphology of pseudounipolar neurons, characterized by a single process proximal to the soma that bifurcates into a peripheral and a central branch. The neurons (large light and small dark) and satellite cells present in DRG, as well as their differentiation, have been described in detail (Pannese, 1974; Hanani, 2005).

Cholinergic markers, such as ChAT and AChE, are expressed in DRGs from early developmental stages (Tata et al., 1994). Acetylcholine promotes the expression of specific neuronal markers (e.g., neurofilament proteins, NF) and the outgrowth of neurites via the activation of the ACh muscarinic receptor (Tata et al., 2003). Moreover, the DRG neurons express ChAT, AChE, and the ACh vesicular transporter (VAChT), and they release ACh during development and in adult (Tata et al., 2004; Bernardini et al., 2004). These data are important not only for the elucidation of sensory neuron development but also for the modulation of sensory perception. Notably, electrophysiological studies have demonstrated

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that muscarinic receptor activation modulates nociception stimuli by regulating CGRP release from the DRG neurons (Bernardini et al., 2001a, 2001b).

In this study, we focused our investigation on the DRG developmental phase, characterized by processes that are related to the final stages of neuronal differentiation. This phase occurs after the contacts with central and peripheral targets have been established in a previous phase, as described by Hamburger and Levi-Montalcini (1949). Our investigation addressed the three following important DRG neuron-related issues: a) the site of ACh release (to address this issue, we cultured DRGs in Campenot chambers, which allow the isolation of the soma and fibers in separate compartments); b) the expression of mediatophore, a transmembrane protein capable of the ACh translocation across the plasma membrane, independently of vesicle-based ACh release, that has been described previously in the vertebrate neurons (Israel and Dunant, 1999) and in other cell types (Fujii et al., 2012); and c) the levels of expression of the muscarinic receptor subtypes.

Materials and methods

Animals

Chick embryos were handled in accordance with the guidelines of the European Communities Council Directive (86/609/EEC of 24 November 1986) and the Italian National law DL/116/92. After decapitation of the embryos, DRGs were dissected, collected, rapidly frozen and stored at −80 °C for subsequent mRNA analysis. For the *in vitro* studies, the DRG neurons dissected from the E12 embryos were immediately placed in cultures as described below.

The developmental stages were established and defined according to the tables detailed by Hamburger and Hamilton (1951). DRG neurons collected on days E12 (stage 32) and E18 (stage 44) of development and day 7 after hatching (P7) were used. The DRG neurons were dissected and pooled from at least 10 to 20 embryos/young chicks from each stage, depending on the type of analysis.

DRG explants

Campenot chambers were assembled in 35 mm collagen-coated dishes after parallel grooves had been made on the collagen in order to guide fiber growth, as described previously (Campenot, 1977) (Fig. 1). The chambers were sealed onto the dishes with a thin layer of silicone to maintain the partition between the central and lateral compartments. Modified Eagle's medium (MEM) supplemented with 5% fetal bovine serum (FBS) and 0.6% Methocel (Sigma) was added to the central chamber and incubated for 2–3 h at 37 °C. Next, medium was added to the lateral compartments and the dishes maintained at 37 °C overnight. DRGs dissected from E12 embryos were then plated as explants into the central compartment. The explants were maintained in culture for 7 days to allow neurite extension into the lateral compartments, as guided by the grooves on the collagen.

ACh release

ACh release was evaluated using the choline oxidase chemiluminescent procedure (Israel and Lesbats, 1981). The medium was removed and each compartment was washed with a saline solution (136 mM NaCl, 5.6 mM KCl, 1.2 mM, $MgCl_2$, 6 mM $CaCl_2$ in 10 mM Tris buffer, pH 8.6) in order to remove pre-existing traces of choline in the medium.

Saline solution (250 µl) was added to each compartment and the dishes were incubated at 37 °C for three consecutive periods (5 min each); during the second incubation period, KCl (final concentration, 80 mM) was added to trigger ACh release. Saline solution (250 µl) was collected before, during, and after stimulation and added to a reaction mixture (500 µl) containing 10 µl luminol (1 mM stock solution),

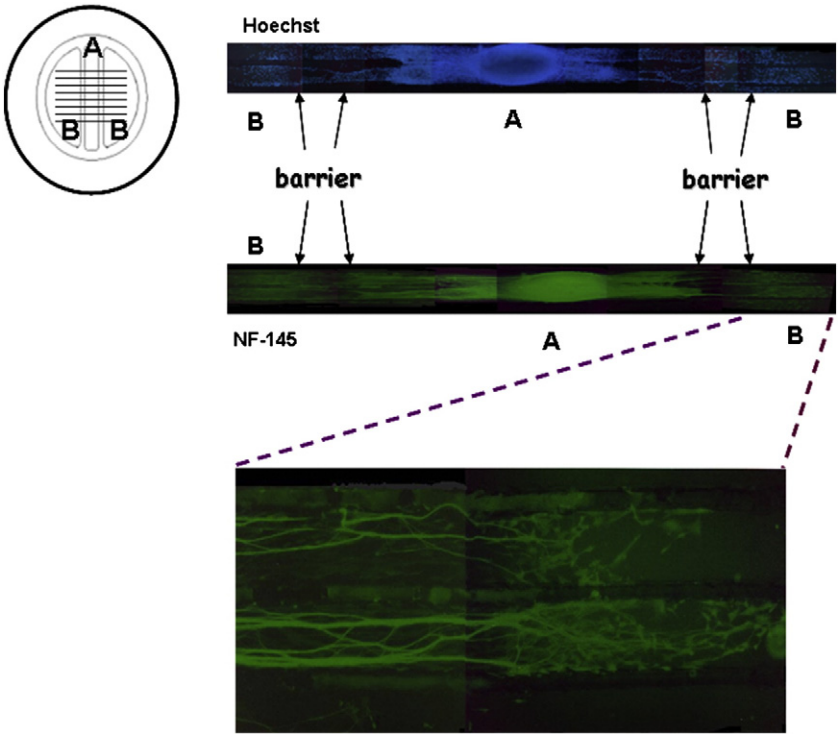

Fig. 1. DRG explants plated in Campenot chambers: (top left) is a schematic representation of a Campenot chamber, where (A) indicates the central chamber and (B) indicates the lateral chambers. Shown in the center is a photographic field of the E12 DRG explants plated in the central chamber and fibers present in the lateral compartments. The nuclei are labeled with Hoechst 33258. Shown in the middle are the neurons and fibers labeled with an α-NF145 primary antibody and a FITC-conjugated secondary antibody-FITC conjugated (25×). Shown at the bottom is a higher magnification of the lateral compartment where the fibers are more evident (160×).