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Direct single cell determination of nitric oxide synthase related metabolites in identified nitrergic neurons

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Abstract

The biochemical characterization of individual nitrergic (NO releasing) neurons is a non-trivial task both in vertebrate and invertebrate preparations. In spite of numerous efforts, there are limited data related to intracellular concentrations of essential metabolites involved in NO synthesis and degradation. This situation creates controversies in both identification of nitrergic neurons and the selection of reliable reporters of NOS activity in heterogeneous cell populations. We take advantage of identified neurons from the pulmonate mollusc *Lymnaea stagnalis* to perform direct single cell microanalysis of intracellular concentrations of the major nitric oxide synthase (NOS) related metabolites such as arginine, citrulline, argininosuccinate, NO_2^-, and NO_3^-. Capillary electrophoresis protocols have been developed to quantitate levels of these metabolites in single identified neurons from the buccal, cerebral, and pedal ganglia using laser-induced fluorescence and conductivity detection. The limits of detection (LODs) for arginine (Arg) and citrulline (Cit) are 84 amol (11 nM) and 110 amol (15 nM), respectively, and LODs for NO_2^- and NO_3^- are <200 amol (<10 nM) each. We report that intracellular concentrations of NOS related metabolites are in the millimolar range and less than 1% of a single cell is required for microchemical analysis. From four cell types tested, only the esophageal motoneuron B2 contains active NOS, and they also contain surprisingly high nitrite levels (up to 5 mM) compared to other neurons tested (peptidergic B4, dopaminergic RPeD1, and serotonergic CGC). These B2 neurons also exhibit an Arg/Cit ratio susceptible to the selective NOS inhibitor L-iminoethyl-*N*-ornithine whereas others neurons do not even though they all may contain NOS transcripts. On the contrary, we found that absolute concentrations of other NOS related metabolites including nitrates are not reliable markers of NOS activity and demonstrate the need for multiple assays for NOS activity.
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1. Introduction

Nitrergic neurons are distinct from other neuronal populations in their ability to produce the gaseous, potentially toxic, messenger nitric oxide (NO) by action of the NO synthase (NOS) enzyme in response to elevated intracellular Ca^{2+} [1–9]. These neurons are also considered to be one of the neuronal groups resistant to various forms of neurodegeneration by unknown mechanisms [10–12]. In spite of numerous efforts, this is a poorly characterized population of cells; there are limited data related to intracellular concentrations of essential metabolites involved in NO synthesis and degradation. Information on the absolute concentrations of

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these metabolites is crucial for pharmacological regulation of NO synthesis by competitive antagonists of NOS [13], as well as for identification of putative nitrergic (NO releasing) cells [14]. Most available data came from either indirect assay or from calculated cellular concentrations of multicellular samples [15]; these data may not be readily applied to neuronal tissues consisting of numerous chemically and physiologically unique cells. In general, NOS-containing cells are diffusely distributed both in the central nervous system (CNS) and in peripheral tissue [16], which prevents them from being reliably recognized and isolated for further electrophysiological or biochemical analysis. Thus, neuron-specific analysis of NO related metabolites is an important but unrealized goal. To overcome obvious difficulties in the characterization and handling of vertebrate neurons we have used the simpler nervous systems of invertebrate animals for such analysis [17,18].

The use of molluscan preparations facilitates single cell isolation/identification, where hundreds of relatively large neurons (some with >200μm soma) can be reliably identified in a variety of gastropod species [8,18]. A molluscan NOS has recently been cloned from the freshwater pulmonate snail, *Lymnaea stagnalis* [19], opening a unique opportunity to directly probe nitrergic neurons. Still, the distribution of this NOS isoform in the *Lymnaea* CNS has not been analyzed in detail, especially in reference to the buccal ganglion, and mapping of nitrergic neurons in this species is primarily based on earlier histochemical data [20,21]. Furthermore, there is no clear correlation between histochemical and molecular data.

In the cerebral ganglion, Korneev et al. [19] used in situ hybridization to show that the cerebral giant cells (CGC), a bilaterally symmetrical pair of feeding modulatory neurons, express an NOS transcript. In a subsequent series of experiments, these authors [22] found a second NOS isoform in the CGC neurons. This isoform contained a sequence of nucleotides that was antisense to the presumed functional NOS sequence, thus they proposed a mechanism of NOS protein synthesis suppression by natural antisense RNA from an NOS pseudogene [22]. As a result of this hypothesis, it appears that some neurons express a variant form of NOS, but may lack the functional enzyme, but this hypothesis has not been proven by direct biochemical measurements.

It may be a common feature that a neuron may contain NOS or protein and be labeled a 'nitrergic' cell while never producing NO. For example, here we show that the CGC neurons identified as containing NOS transcripts do not appear to have functional NOS. Thus, direct measurements of NOS activity are imperative for functional identification of nitrergic neurons. A direct microchemical assay of putative nitrergic neurons can be accomplished by employing capillary electrophoresis (CE) separation technology with nanoliter injection volumes and nanomolar detection limits [23–28] which is ideally suited for single cell microanalysis of NOS related metabolites [17].

Several protocols have been developed for such CE microanalysis and have been successfully used in experiments on identified neurons isolated from a marine mollusc, *Pleurobranchaea californica* [29–31]. Unfortunately, NOS itself has not been cloned from *Pleurobranchaea*; therefore, a direct correlation between the presence of NOS transcripts and enzymatic NOS activity has not been established. Here, we address these questions using well-characterized neurons from *Lymnaea stagnalis*.

2. Experimental

2.1. Animals

Specimens of the freshwater pond snail, *Lymnaea stagnalis*, were obtained from stock raised at the aquaculture facilities at the Vrije Universiteit, Amsterdam, and maintained in aerated fresh water aquaria under constant light. The animals were fed lettuce twice weekly. All animals selected for further experimentation were adults at least 25 mm in shell length.

2.2. Cell isolation, cell culture and histochemistry

All experiments were performed on identified neurons isolated from the CNS of *Lymnaea stagnalis*. Neurons were visually identified according to earlier published maps and electrophysiological characteristics [32–36] and individually removed from the intact CNS following recently reviewed protocols [37]. Briefly, central ring ganglia were removed from intact animals submerged in saline (mM: NaCl 40.0, KCl 1.7, $CaCl_2$ 4.1, $MgCl_2$ 1.5, Hepes (*N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid 10.0)), pH 7.8) and subjected to a brief enzymatic treatment (trypsin type III, 2 mg/mL) in defined media (DM). DM consisted of serum-free 50% (v/v) Liebowitz's L-15 media (formula #82-5154EL, GIBCO, Grand Island, NY) with additional salts (mM: NaCl 40.0, KCl 1.7, $CaCl_2$ 4.1, $MgCl_2$ 1.5, Hepes 10.0), 10 mM glucose, 1.0 mM L-glutamine, and 20 μg/mL gentamycin with the final pH adjusted to 7.9 with 1 N NaOH. Following enzyme treatment, ganglia were pinned down in a dissection dish containing high osmolarity DM (additional 40 mM glucose), and both the outer and inner connective tissue sheaths surrounding the ganglia were removed mechanically using fine tipped forceps. Individual neuronal somata were visually identified and selectively isolated by applying gentle suction via a microsyringe (Gilmont, GS 110, Barrington, IL) attached to a fire polished, silicone coated (Sigmacote,

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