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Research Report

Increase of Kv3.1b expression in avian auditory brainstem neurons correlates with synaptogenesis *in vivo* and *in vitro*

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ABSTRACT

In the auditory system voltage-activated currents mediated by potassium channels Kv1.1 and Kv3.1b and their interaction with sodium inward currents play a crucial role for computational function. However, it is unresolved how these potassium channels are developmentally regulated. We have therefore combined a biochemical investigation of Kv1.1 and Kv3.1b protein expression with electrophysiological recordings of membrane currents to characterize neuronal differentiation in the auditory brain stem of the chick. Differentiation *in vitro* was compared with cells prepared from corresponding embryonic stages *in vivo*. Using a computer model based on the empirical data we were then able to predict physiological properties of developing auditory brain stem neurons. *In vivo* Kv3.1b expression increased strongly between E10 and E14, a time of functional synaptogenesis in the auditory brainstem. We also found this increase of expression *in vitro*, again coinciding with synaptogenesis in the cultures. Whole-cell patch recordings revealed a corresponding increase of the (Kv3.1-like) high threshold potassium current. In contrast, Kv1.1 protein expression failed to increase *in vitro*, and changes in (Kv1.1-like) low threshold potassium current with time in culture were not significant. Electrophysiological recordings revealed that sodium inward currents increased with cultivation time. Thus, our data suggest that Kv3.1b expression occurs with the onset of functional synaptogenesis, while a different signal, absent from cultures of dissociated auditory brain stem, is needed for Kv1.1 expression. A biophysical model constructed with parameters from our recordings was used to investigate the functional impact of the currents mediated by these channels. We found that during development both high and low threshold potassium currents need to be increased in a concerted manner with the sodium conductance for the neurons to exhibit fast and phasic action potential firing and a narrow time window of coincidence detection.

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Abbreviations: DIV, days in vitro; E, embryonic day (after fertilization); HTK, high threshold potassium current; Kv, voltage-gated potassium channel subunit; LTK, low threshold potassium current; Nav, voltage-activated sodium current; NL, nucleus laminaris; NM, nucleus magnocellularis

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1. Introduction

Neurons in the auditory system contain voltage-gated potassium channels that shape the subthreshold response, the action potential waveform and the firing pattern (Trussell, 1999; Dodson et al., 2002; Rothman and Manis, 2003; Gittelman and Tempel, 2006), precisely tuned to their computational roles (Fukui and Ohmori, 2004; Kuba et al., 2005). Avian auditory brainstem neurons of the nucleus magnocellularis and nucleus laminaris (NM, NL; the functional analogs of the mammalian ventral cochlear nucleus and medial superior olive) generate high-frequency action potentials that are locked to the stimulus phase (Warchol and Dallos, 1990; Köppl, 1997). Moreover, neuronal calculation rules (Jeffress, 1948; Overholt et al., 1992; Hyson, 2005) involve submillisecond coincidence detection, which demands reliable shunting of non-correlated input (Gerstner et al., 1996; Trussell, 1997; Svirskis et al., 2002; Day et al., 2008). These functions can be attributed to two major types of potassium channels (Reyes et al., 1994; Rathouz and Trussell, 1998). Channels containing Kv1.1 subunits mediate a low-threshold potassium current (LTK; Gutman et al., 2005) which decreases the membrane resistance at subthreshold membrane potentials, thus reduces temporal summation and increases the neuronal signal to noise ratio (Trussell, 1999; Svirskis et al., 2002). Channels containing Kv3.1b subunits mediate a high-threshold potassium current (HTK; Gutman et al., 2005) that increases the repolarization rate of the AP, which facilitates high-frequency firing (Wang et al., 1998; Macica et al., 2003).

Kv1.1 and Kv3.1b subunits are expressed in the adult (Parameshwaran et al., 2001) and embryonic (Zhou et al., 2001; Parameshwaran-Iyer et al., 2003; Feng and Morest, 2006) auditory brainstem of the chicken. Moreover, development of associated currents was shown with voltage-clamp recordings in acute preparations (Hendriks et al., 1999; Howard et al., 2007; Gao and Lu, 2008). These studies indicated that the strongest increase in HTK takes place during the period of functional synaptogenesis and the onset of electrical activity (Jackson et al., 1982).

Upregulation of the LTK appears later in development, accompanying a period of spontaneous bursting of increasing frequency (Lippe, 1994). Although generally believed (Hendriks et al., 1999; Feng and Morest, 2006), it has not yet been demonstrated conclusively, whether there is a causal link between synaptogenesis, bursting activity and potassium channel expression.

In a culture system enriched with embryonic NM and NL neurons we showed before (Kuenzel et al., 2007) that functional synaptogenesis takes place after about 3–7 days *in vitro* (DIV, following preparation at E6.5, virtual age of 10–14 days after fertilization), which was remarkably similar to the situation *in vivo*. In the present study we compare functional expression of Kv1.1 and Kv3.1b protein and associated currents during embryonic development *in vivo* and *in vitro*. With a biophysical model based on these data we evaluate the impact of the observed developmental changes on auditory function. Therefore, this study, which establishes a correlative connection between protein expression and physiological development, explains the logical order in which cellular specialization emerges in the auditory brainstem circuitry.

2. Results

2.1. Time course of calretinin and potassium channel expression in vivo and in vitro

In the avian brainstem the expression of the calcium-binding protein calretinin can be used as a marker for auditory neurons (Parks et al., 1997). Calretinin as well as Kv1.1 and Kv3.1b protein expression was evaluated with SDS-PAGE and Western blotting (Fig. 1A). Protein isolates from the auditory region of the chicken brainstem excised at E7, 10, 14 and 20 were compared to protein isolates from cultures prepared from the same area at E6.5 and cultured for corresponding time intervals (E6.5 plus DIV1, 3, 7, 14). To demonstrate relative changes of protein expression during development all data were normalized to the expression values at E7 (*in vivo*) or E6.5+1 DIV (*in vitro*). Specificity of the antibodies directed against Kv1.1 and Kv3.1b in the chicken system was tested by preincubation with the antigen peptide as delivered by the manufacturer. No immunoreactive bands were seen after this procedure (data not shown).

The calretinin band had a molecular weight of 30 kDa, corresponding to its published size (Stevens and Rogers, 1997). *In vivo*, a twofold increase of expression between E7 and E10 (1.9±0.5; mean±SEM) was detectable (see Fig. 1B). Expression remained high up to E14 (2.1±0.62) and slightly decreased between E14 and E20 (1.8±0.53) although the changes fell just short of the required level of significance ($N=3$ independent experiments; Kruskal–Wallis test). In the cell culture, a significant change in calretinin expression over time was found. Between DIV1 and DIV7 (1.36±0.08) the expression showed only a minor increase in calretinin expression. However, between DIV7 and DIV14 (0.78±0.13) the expression was significantly reduced to about 75% of initial levels ($N=5$, Kruskal–Wallis $p<0.05$, $H=9.77$; *post-hoc U*-test $p<0.05$).

The immunoreactive band of potassium channel Kv1.1 (Fig. 1C) had a molecular weight of 87 kDa and was detectable at all stages *in vivo* and *in vitro*. While a small but constant increase of expression during *in vivo* development was seen (E10: 1.15±0.16; E14: 1.26±0.2; E20: 1.37±0.15), Kv1.1 signal levels remained unchanged (DIV3: 1.1±0.04) or slightly decreased (DIV7: 0.8±0.04; DIV14: 0.78±0.13) *in vitro*. Neither the changes of expression in the *in vivo* ($N=4$) nor in the *in vitro* ($N=5$) data reached significance levels (Kruskal–Wallis test).

We detected the Kv3.1b (see Fig. 1D) protein in a band corresponding to 92 kDa molecular weight. During *in vivo* development there was a significant change of expression over time ($N=4$, Kruskal–Wallis $p<0.05$, $H=10.61$). While expression levels remained low from E7 to E10 (0.9±0.3), a steep significant increase was detectable between E10 and E14 (5.3±0.7; *post-hoc U*-test, $p<0.05$). After that, expression levels remained high up to E20 (5.2±1.3). This significant change in expression was also present *in vitro* ($N=5$, Kruskal–Wallis $p<0.05$, $H=9.38$) at corresponding points in time. Here, expression levels were initially low (DIV3: 1.3±0.8). At DIV7 (corresponding to *in vivo* E14) the Kv3.1b signal levels were found to be significantly increased (4.9±0.5; *post-hoc U*-test, $p<0.05$). As in the *in vivo* time series, expression levels remained constantly high after this increase (DIV14: 5.1±1.6).