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DEVELOPMENTAL ROLES OF THE SPONTANEOUS DEPOLARIZATION WAVE IN SYNAPTIC NETWORK FORMATION IN THE EMBRYONIC BRAINSTEM

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Abstract—One of the earliest activities expressed within the developing central nervous system is a widely propagating wave-like activity, which we referred to as the depolarization wave. Despite considerable consensus concerning the global features of the activity, its physiological role is yet to be clarified. The depolarization wave is expressed during a specific period of functional synaptogenesis, and this developmental profile has led to the hypothesis that the wave plays some roles in synaptic network organization. In the present study, we tested this hypothesis by inhibiting the depolarization wave in ovo and examining its effects on the development of functional synapses in vagus nerve-related brainstem nuclei of the chick embryo. Chronic inhibition of the depolarization wave had no significant effect on the developmental time course, amplitude, and spatial distribution of monosynaptic excitatory postsynaptic potentials in the first-order nuclei of the vagal sensory pathway (the nucleus of the tractus solitarius (NTS) and the contralateral non-NTS region), but reduced polysynaptic responses in the higher order nucleus (the parabrachial nucleus). These results suggest that the depolarization wave plays an important role in the initial process of functional synaptic expression in the brainstem, especially in the higher order nucleus of the cranial sensory pathway.

Key words: Optical recording, voltage-sensitive dye, spontaneous activity, chick embryo, vagal nucleus, synaptic network formation.

INTRODUCTION

Spontaneous activity is expressed in the developing central nervous system well before sensory pathways are functionally organized, and is considered to play a fundamental role in neural development (Feller, 1999; O'Donovan, 1999; Roerig and Feller, 2000; Chatonnet et al., 2002; Moody and Bosma, 2005; Blankenship and Feller, 2010). Using optical recordings with voltage-sensitive dyes, we previously demonstrated that one of the earliest spontaneous activities consists of a large-scale wave of neural depolarization, which propagates over a wide region of the central nervous system including the spinal cord, brainstem, cerebellum, and forebrain (Momose-Sato et al., 2007, 2009, 2012a; for reviews see Momose-Sato and Sato, 2013, 2016a). We referred to this widely spreading optical wave as the depolarization wave.

The depolarization wave has been observed in several different animals including chicks, rats, and mice, and is considered to be analogous to the rhythmic episodes of spontaneous bursting activity recorded electrophysiologically (Di Pasquale et al., 1992; Fortin et al., 1995; Milner and Landmesser, 1999; Abadie et al., 2000; Hanson and Landmesser, 2003; for reviews see Hanson et al., 2008; Momose-Sato and Sato, 2013). Despite considerable consensus concerning the global features of the activity, its physiological role, especially the significance of large-scale propagation, is yet to be clarified. The depolarization wave is expressed during a specific period of embryogenesis, with synchronization throughout the brain and spinal cord being observed at E (embryonic day: days of incubation in avians and days of pregnancy in mammals) 4–8 in chicks and E11–13 (and in some E14 preparations) in mice (Momose-Sato et al., 2009, 2012a; Momose-Sato and Sato, 2014). During these stages, synaptic contacts are established and become functional between cranial/spinal sensory nerves and postsynaptic neurons in the brain/spinal cord (Lee et al., 1988; Mochida et al., 2001b; Momose-Sato et al., 2001a, 2015; Glover et al., 2008). The late phase of synaptogenesis, including the refinement and detailed patterning of connections, is known to be activity-dependent (Goodman and Shatz, 1993; Katz and Shatz, 1996; Hua and Smith, 2004). However, this process seems not to be the primary target of the depolarization wave, since the wave is most prominent during the initial stage of functional synaptic expression and disappears

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Abbreviations: APV, $_{DL}$-2-amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DMNV, dorsal motor nucleus of the vagus nerve; E, embryonic day (days of incubation in avians and days of pregnancy in mammals); EPSP, excitatory postsynaptic potential; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-$_{D}$-aspartate; NTS, nucleus of the tractus solitarius; PBN, parabrachial nucleus.

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thereafter, after which it is replaced by activity localized to the spinal or respiratory network (Momose-Sato et al., 2009, 2012a; Momose-Sato and Sato, 2014). This developmental profile has led to the hypothesis that the depolarization wave regulates the early phase of synaptic network organization.

In the present study, we tested this hypothesis by inhibiting the depolarization wave in ovo and examining its effects on functional synaptic expression in the brainstem. Chronic blockade of activity by the in ovo application of drugs has been frequently used to study the functional significance of activity during embryogenesis. This method has advantages in that it is technically easy to employ and that receptor functions can be blocked only during a specific period of development. In earlier studies, neuromuscular blocking agents were used to study their effects on the development of muscles and bones (Roufa and Martonosi, 1981; Persson, 1983; Hall and Herring, 1990) and of spinal motoneurons (Dahm and Landmesser, 1991; Martin-Caraballo and Dryer, 2002; Oppenheim et al., 2008). In more recent studies, non-cholinergic blockers were applied, and the results suggested that correlated activity generated in the spinal cord plays an important role in the formation of spinal motor circuits (Hanson and Landmesser, 2004; Gonzalez-Islas and Wenner, 2006; Wilhelm et al., 2009; Kastanenka and Landmesser, 2010).

In the present study, the in ovo application of drugs was used for the first time to examine the role of correlated activity along the cranial sensory pathway. Immature neurons in the developing brain are small and fragile, and thus conventional electrophysiological tools such as microelectrodes are difficult to use. We have overcome this obstacle by applying an optical recording technique with voltage-sensitive dyes and revealed the developmental processes of synaptogenesis in the brain and spinal cord (for reviews see Momose-Sato et al., 2001a, 2015; Glover et al., 2008). Here, we optically identified brainstem nuclei and examined the effects of chronic inhibition of the depolarization wave on the functional expression of postsynaptic potentials.

Preliminary results have appeared in abstract form (Momose-Sato and Sato, 2017).

EXPERIMENTAL PROCEDURES

Experiments were approved by the Ethics Committees of Kanto Gakuin University and Komazawa Women's University and were performed in accordance with the Japan Society for the Promotion of Science guidelines for the care and use of laboratory animals.

In ovo application of the blockers: Fertilized eggs of White Leghorn chickens (Shiraishi Laboratory Animals, Saitama, Japan and Nippon Bio-Supply Center, Tokyo, Japan) (n = 198 for control, n = 359 for bicuculline/strychnine experiments, n = 294 for *d*-tubocurarine experiments, n = 73 for Ringer application in ovo) were incubated at 38 °C and 60% humidity. In the chick embryo, the widely propagating correlated activity, termed the depolarization wave, appears in the brainstem from E4 (4 days of incubation) to E8 (Momose-Sato et al., 2009; Momose-Sato and Sato, 2014). The depolarization wave is mediated by nicotinic acetylcholine receptors at early stages (∼E6) and glutamate receptors at later stages (E6∼), and also depends on excitatory actions of GABA (γ-aminobutyric acid) and glycine at every stage (Mochida et al., 2009). In the present study, we used a mixture of GABA- and glycine-receptor antagonists to block the depolarization wave during E4-E8 and *d*-tubocurarine to block the early wave. At E4, a window in the shell was opened to allow drug application. A mixture of bicuculline methobromide and strychnine (Sigma Chemical Co., St. Louis, MO, USA) or *d*-tubocurarine alone (Wako Pure Chemical Industries, Osaka, Japan) [500 μM–5 mM (usually 5 mM) in 100 μl of Ringer's solution containing penicillin (100 unit/ml)/streptomycin (0.1 mg/ml)] was administered onto the chorioallantoic membrane of the embryo. After administration of the blockers, the shell window was sealed with paraffin film, and the incubation was continued. We applied the blockers daily from E4 until the day before the optical recording.

Preparation for optical recording: In the present study, we targeted sensory nuclei of the vagus nerve, since functional synaptogenesis has been most extensively analyzed in this system (for reviews see Momose-Sato and Sato, 2006, 2011, 2015). At E6 or E8, the embryos were isolated and decapitated, and *en bloc* brainstem preparations with the vagus nerve attached were dissected. Slice preparations of about 1500 μm were made by transecting the isolated brainstems at the level of the vagus nerve root. The preparations were kept in Ringer's solution, which contained (in mM) NaCl, 138; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.5; glucose, 10; and Tris–HCl buffer (pH 7.3), 10. In the Mg^{2+}-free experiment, $MgCl_2$ was replaced with $CaCl_2$. The solution was equilibrated with oxygen. The meningeal tissue was removed, and the preparations were stained with a voltage-sensitive merocyanine-rhodanine dye, NK2761 (0.2 mg/ml, 10- to 15-min staining) (Hayashibara Biochemical Laboratories Inc./Kankoh-Shikiso Kenkyusho, Okayama, Japan: Kamino et al., 1981; Salzberg et al., 1983; Momose-Sato et al., 1995). This dye is particularly useful in embryonic nervous and cardiac tissues (Kamino, 1991; Momose-Sato et al., 1995, 2015). After the staining, each preparation was placed in a recording chamber with the ventral side (for *en bloc* preparations) or spinal cord side (for slice preparations) facing up by pinning it with tungsten wires. The preparation was continuously superfused with the bathing solution at 1–2 ml/min at room temperature (24–28 °C) except for at the time of data acquisition.

Electrical stimulation of the vagus nerve: The vagus nerve was stimulated with a glass microsuction electrode to evoke postsynaptic responses in the vagal sensory nuclei. Depolarizing square current pulses (8 μA/5 ms), which evoked the maximum response, were applied to the vagus nerve with a single shot. To examine the pharmacological nature of the vagal postsynaptic responses, glutamatergic antagonists, DL-2-amino-5-phosphonovaleric acid (APV) (Sigma Chemical Co., St. Louis, MO, USA) and 6-cyano-7-nitroquinoxa

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