Experimental Neurology 289 (2017) 117–127

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Alterations in hypoglossal motor neurons due to GAD67 and VGAT deficiency in mice

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ARTICLE INFO

Article history:
Received 11 August 2016
Received in revised form 25 November 2016
Accepted 8 December 2016
Available online 10 December 2016

Keywords:
Motor neuron
Brainstem
Synaptic currents
Dendrites
Spines

ABSTRACT

There is an emerging body of evidence that glycinergic and GABAergic synaptic inputs onto motor neurons (MNs) help regulate the final number of MNs and axonal muscle innervation patterns. Using mutant glutamate decarboxylase 67 (GAD67) and vesicular inhibitory amino acid transporter (VGAT) deficient mice, we describe the effect that deficiencies of presynaptic GABAergic and/or glycinergic release have on the post-synaptic somatodendritic structure of motor neurons, and the development of excitatory and inhibitory synaptic inputs to MNs. We use whole-cell patch clamp recording of synaptic currents in E18.5 hypoglossal MNs from brainstem slices, combined with dye-filling of these recorded cells with Neurobiotin™, high-resolution confocal imaging and 3-dimensional reconstructions. Hypoglossal MNs from GAD67- and VGAT-deficient mice display decreased inhibitory neurotransmission and increased excitatory synaptic inputs. These changes are associated with increased dendritic arbor length, increased complexity of dendritic branching, and increased density of spiny processes. Our results show that presynaptic release of inhibitory amino acid neurotransmitters are potent regulators of hypoglossal MN morphology and key regulators of synaptic inputs during this critical developmental time point.

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1. Introduction

During development, the mammalian neuromotor system undergoes a period of programmed motor neuron (MN) cell death. Over 50% of all motor neurons generated during neurogenesis are lost in the final trimester in utero (Lance-Jones, 1982; Oppenheim, 1991). The final number of surviving MNs is regulated in an activity-dependent manner, as reduced skeletal muscle activity causes increased MN survival and neuromuscular innervation (Landmesser, 1992; Banks et al., 2003), whereas increased skeletal muscle activity causes decreased MN survival and neuromuscular innervation (Oppenheim and Nunez, 1982). These alterations in muscle activity depend on nervous system activity, that is regulation of MN firing by other central neurons (neuronal activity), which is then passed onto muscle via neuromuscular synapses (Banks and Noakes, 2002).

Glycine and GABA are important inhibitory neurotransmitters in the adult nervous system, and they are vital for normal postnatal neuromotor function and development, as GAD67 and VGAT-deficient mice die within a few hours after birth from respiratory complications and failure to suckle (Asada et al., 1997; Wojcik et al., 2006; Kakizaki et al., 2015). GAD67 is the major synthesizing enzyme for GABA in prenatal and neonatal mice, and electrophysiological studies have shown that GABAergic neurotransmission is reduced or nearly eliminated in brainstem respiratory neurons from GAD67 mutants (Fujii et al., 2007). VGAT is the transporter protein which transfers both GABA and glycine into presynaptic vesicles, and both GABAergic and glycinergic neurotransmission are absent or severely reduced in spinal cord neurons (Wojcik et al., 2006), brainstem respiratory neurons (Fujii et al., 2007), spinal MNs (Saito et al., 2010) and hypoglossal MNs (Rahman et al., 2015) of VGAT mutants.

However, during neural development, GABA and glycine neurotransmission are thought to provide important synaptic sources of depolarizing chloride ion (Cl^-) conductances to MNs (Nishimaru et al., 1996; Singer and Berger, 2000; Ben-Ari, 2002). This response is due to the high intracellular Cl^- concentration in foetal and neonatal neurons causing membrane depolarization as Cl^- exits to the extracellular space when Cl^- permeable channels gated by glycine and GABA are activated. Closer to birth, or during early postnatal life, the action

Abbreviations list: E, embryonic day; EPSC, excitatory postsynaptic current; GAD67, glutamate decarboxylase 67; IPSC, inhibitory postsynaptic current; MN, motor neuron; VGAT, vesicular inhibitory amino acid transporter; WT, wild-type.

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http://dx.doi.org/10.1016/j.expneurol.2016.12.004
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of GABA and glycine shifts from depolarizing to hyperpolarizing or inhibitory as the intracellular Cl^- concentration decreases, resulting in movement of Cl^- into the intracellular compartment upon activation by glycine and/or GABA (Akerman and Cline, 2007), leading to a refinement of the MN inputs to target skeletal muscle. Morphological and physiological studies of mice lacking the glycine receptor-clustering molecule Gephyrin (Kneussel et al., 1999) support this mechanism, as loss of glycinergic neurotransmission leads to increased MN activity and decreased MN survival when compared to wild-type controls (Banks et al., 2005), suggesting that the effect of GABAergic and glycinergic neurotransmission onto hypoglossal MNs is inhibitory at birth.

However, the mechanisms by which increased MN activity leads to greater MN loss, compared to wild-type MNs (Banks et al., 2005; Fogarty et al., 2013b; Fogarty et al., 2015b) remains unclear. One mechanism underlying increased MN loss may be the effects of an imbalance between excitatory and inhibitory inputs onto mutant MNs overloading embryonic MNs with glutamate, a known mediator of excitotoxic neuronal death (Choi, 1992, 1995), and of dendritic growth (Kalb, 1994; Inglis et al., 2002; Metzger, 2010; Koleske, 2013), and increased motor neuron excitability (van Zundert et al., 2008). By quantifying synaptic activity and morphology of individual MNs from mice with deficiencies in GABA and/or glycinergic neurotransmission, we may thus gain an understanding of how altered central synaptic inputs onto MNs regulate final hypoglossal MN numbers at birth, when the neuromotor system must be functional at birth for suckling and respiration.

This study therefore aimed to quantify alterations to the dendritic arbor, spine density, and functional synaptic inputs of hypoglossal MNs at E18.5, using GAD67- and VGAT-deficient mice, compared to wild-type (WT) MNs. We used patch-clamp electrophysiology techniques to record synaptic activity, followed by dye-filling of the recorded MNs with Neurobiotin™ for subsequent high resolution morphometric measurements. We also confirm our previous qualitative observations of decreased IPSC frequency and increased EPSC frequency of GAD67-deficicient XI MNs and increased somatic spine density in VGAT XII MNs compared to WT controls (Kanjhan et al., 2016b).

2. Materials and methods

2.1. Animals used and ethical statement

We used five GAD67-deficient mice ($GAD67^{-/-}$), seven VGAT-deficient mice ($VGAT^{-/-}$) and nine wild-type mice (WT) at embryonic day 18/postnatal day 0 (termed E18.5). $GAD67^{-/-}$ and $VGAT^{-/-}$ mice and their respective WT littermates were generated and genotyped in accordance with previous studies (Tamamaki et al., 2003; Saito et al., 2010). All mice including WT littermates were derived from heterozygote $GAD67^{+/-}$ or heterozygote $VGAT^{+/-}$ breeding scheme on a C57Bl/6J genetic background. This embryonic age was chosen as it is when both mutants display decreased MN numbers compared to controls (Fogarty et al., 2013b, 2015b). By contrast neither mutant shows significant differences in hypoglossal nucleus gross morphology compared to controls (Fogarty et al., 2013b, 2015b). In these age groups, immediately before and after birth, the morphological properties of hypoglossal MNs do not change in WT mice (Kanjhan et al., 2016a). All procedures were approved by the University of Queensland Animal Ethics Committee (Permit Numbers: 227-09, 924-08) and complied with ethical guidelines for animal experimentation (Drummond, 2009). Our experiments were conducted in accordance with the Queensland Government Animal Research Act 2001 and Protection Regulations (2001 and 2008) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th Edition (National Health and Medical Research Council, 2013) https://www.nhmrc.gov.au > guidlines.

2.2. Slice preparation and electrophysiological recording

Mice were sacrificed immediately after birth (P0) or harvested as E18 embryos from time-mated pregnant females. Pregnant females were killed by cervical dislocation and embryos were removed by cesarean section. Pups/embryos were anesthetized by hypothermia in an ice-cold dissection tray. Dissection and brain slicing was carried out as previously detailed (Fogarty et al., 2013a; Kanjhan and Bellingham, 2013; Kanjhan et al., 2016a).

Patch electrodes were pulled from borosilicate glass capillaries (Vitrex Modulohm, Edwards Medical, Narellan, NSW) to give a tip resistance of 3–4 MΩ when filled with intracellular solution containing (in mM): 135 Cs^+MeSO_4, 6 KCl, 1 EGTA, 2 $MgCl_2$, 5 Na-HEPES, 3 ATP-Mg^{2+}, 0.3 GTP-Tris (pH 7.25, osmolarity 305 ± 5 mOsm) (Bellingham and Berger, 1996). The pipette was then back-filled with 1–2 μl of intracellular solution containing 2% Neurobiotin™ (NB, Vector Labs). Recording procedures and single cell dye-electroporation parameters were unchanged from previous studies (Fogarty et al., 2013a; Kanjhan and Bellingham, 2013; Kanjhan et al., 2016a; Fogarty et al., 2016). After the membrane seal was ruptured by voltage pulses or suction, spontaneous IPSCs were recorded at a holding membrane potential of 0 mV and spontaneous EPSCs were recorded at a holding potential of −60 mV; we have previously shown that these recording conditions produced a reversal potential for IPSCs which was equivalent to a negative calculated Cl^- reversal potential, and a reversal potential for EPSCs which was equivalent to a positive mixed cation reversal potential (Fogarty et al., 2013a; Kanjhan and Bellingham, 2013) and only cells with a series resistance of below 25 mΩ and a capacitance of <40 pF were included in the electrophysiology data set (van Zundert et al., 2008). Spontaneous synaptic events were detected over 75 s of continuous recording, chosen randomly from 2-minute epochs, using Axograph X (Axograph Scientific). Peak-to-peak noise ranged from 2 to 10 pA, and only events with peak amplitude > peak-to-peak noise were analyzed.

2.3. Immunocytochemistry, imaging and morphologic quantification

After filling, slices were left in the recording chamber for 6–12 min to allow NB diffusion (Fogarty et al., 2013a; Kanjhan and Bellingham, 2013), then removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline pH 7.4 (PBS) for 30 min, washed in PBS and incubated for 4 h in PBS containing 4% bovine serum albumin (BSA) and 0.05% Triton-X 100 at 4 °C. Slices were then incubated for 4 h at 4 °C in Cy3-Streptavidin (Sigma; 1:500 in 4%BSA PBS) to visualize NB, as detailed previously (Kanjhan and Vaney, 2008; Kanjhan and Bellingham, 2013; Fogarty et al., 2013a). Slices were washed in PBS and mounted on slides.

Low-powered (20× objective) *z*-series 0.8 NA (with a 1.0 μm *z*-step) from a Zeiss LSM 510 META scanning confocal microscope (Carl Zeiss, Gottingen, Germany) using 555 nm laser excitation/detection filters were used to create confocal stacks of the MN and its entire dendritic arbor. Morphological properties of NB-filled hypoglossal MNs were analyzed from these confocal images using Neurolucida™ (MBF Bioscience Inc.) as previously detailed (Fogarty et al., 2015a). For quantifying spine density, a high-powered (63× oil) objective (1.4 NA) was used with a 2.5× zoom to collect *z*-series with a 0.33 μm *z*-step. Dendritic processes were classified as spines only if they were <3 μm long and <0.8 μm in cross-sectional diameter (Harris, 1999; Fogarty et al., 2015a). A total dendrite length of 115,004 μm was traced for morphometric analysis in 41 neurons (15 WT, 11 $GAD67^{-/-}$ and 15 $VGAT^{-/-}$ mice). The gross morphology of the hypoglossal nucleus was measured using the Cavalieri technique in a manner identical to previous studies (Fogarty et al., 2013b, 2015b, 2016).

2.4. Statistical analysis

Data were analyzed with Prism 6 (Graphpad) and expressed as mean ± standard error of the mean (s.e.m.). Statistically significant changes were determined using a one-way ANOVA, with Tukey's post-test, or a two-way ANOVA with a Bonferroni post-test, where

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