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Changes in the expression and localization of signaling molecules in mouse facial motor neurons during regeneration of facial nerves

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ABSTRACT

After injury, peripheral axons usually re-extend toward their target, and neuronal functions recover. Previous studies have reported that expression of various molecules are transiently altered in motor neurons after nerve injury, but the time course of these changes and their relationship with functional recovery have not been clearly demonstrated. We used the mouse facial nerve transection and suturing model, and examined the changes in expression of five molecules, choline acetyl transferase (ChAT), galanin, calcitonin gene-related protein (CGRP), gephyrin, and potassium chloride co-transporter 2 (KCC2) in the facial motor neurons after surgery until recovery. Number of ChAT-positive neurons was markedly decreased at days 3 and 7, and recovered to the normal level by day 60, when facial motor functions recovered. Localization of two neuropeptides, CGRP and galanin, was increased in the perikarya and axons during regeneration, and returned to the normal levels by days 60 and 28, respectively. Expression of two postsynaptic elements of γ-amino butyric acid synapses, gephyrin and KCC2, was decreased at days 3 and 7, and recovered by day 60. These results suggest that ChAT, CGRP, and KCC2 may be objective indicators of regeneration, and altering their expression may be related to the functional recovery and axonal re-extension.

1. Introduction

Neuromotor functions are organized by complex inter-neuronal connections. Injury of axons affects neural functions. In the peripheral nervous system, injured axons often re-extend toward their targets, such as skeletal muscles, and neural functions likely recover (Hoffman, 2010; Okano, 2010; Palispis and Gupta, 2017). During this period, the expression and localization of many molecules, such as calcitonin gene-related peptide (CGRP) (Chen et al., 2010; Fukuoka et al., 1999), galanin (Gey et al., 2016; Herdegen and Leah, 1998; Makwana et al., 2010; Stern et al., 2012), interleukin-6 and interleukin-1β (Streit et al., 2000), ionized Ca^{2+} –binding adapter molecule 1 (Ichimiya et al., 2013), growth associated protein-43 (McNamara et al., 2000), and c-Jun (Herdegen and Leah, 1998; Raivich et al., 2004), transiently increase in the motor neurons. In contrast, nitric oxide synthase (Fiallos-Estrada et al., 1993), glycogen synthase (Takezawa et al., 2014), vesicular acetylcholine transporter (Ichimiya et al., 2013; Takezawa et al., 2014), potassium chloride co-transporter 2 (KCC2) (Nabekura et al., 2002; Tatetsu et al., 2012), and choline acetyltransferase (ChAT) (Brecht et al., 1995; Ichimiya et al., 2013; Takezawa et al., 2014; Tatetsu et al., 2012) decrease in expression and localization. These findings suggest that after nerve injury, synthesis of acetylcholine (ACh) may stop in motor neurons, and up- or down-regulation of the above mentioned molecules may contribute to axonal sprouting and re-extension.

In particular, downregulation of KCC2 expression increases intracellular chloride ion concentration ($[Cl^-]_i$), shifts action of γ-amino butyric acid (GABA) from inhibition to excitation, and may induce axonal re-extension (Nabekura et al., 2002; Tatetsu et al., 2012; Toyoda et al., 2003), as observed during normal neuronal development (Shimizu-Okabe et al., 2002; Takayama and Inoue, 2006, 2007, 2010). Nevertheless, the precise timing of these events is not fully understood. Many previous studies have reported the transient changes in expression (increasing or decreasing) during short periods after injury, but the

Abbreviations: 7L, lateral subnucleus of facial nucleus; 7VM, ventromedial subnucleus of facial nucleus; ACh, acetylcholine; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; Cl^-, chloride ion; $[Cl^-]_i$, intracellular chloride ion concentration; Dpi, day post-injury; FMN, facial motor neuron; GABA, γ-amino butyric acid; Gly, glycine; KCC2, potassium chloride co-transporter 2; PB, phosphate buffer; VGAT, vesicular GABA transporter

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Table 1
Antibody characterization.

Antigen	Immunogen	Manufacture species, reference	Dilution
CGRP	Whole antiserum, synthetic CGRP (rat) conjugated to KLH (Fasanella et al., 2008; Ramer, 2008)	Sigma-Aldrich, No. C8198, Lot. 070M4835, rabbit polyclonal (Fasanella et al., 2008; Ramer, 2008)	1:40,000
ChAT	Human placental enzyme	Chemicon Millipore, No. AB144, Lot. LV1449628, goat polyclonal (Lu et al., 2008)	1:3000
Galanin	Synthetic peptide	Peninsula Laboratories International, Inc., No. T-4326, Lot. A14214, rabbit polyclonal	1:20,000
Gephyrin	Purified rat gephyrin	Synaptic system, No. 147011, Lot. 147011/42, mouse monoclonal	1:2000
KCC2	Synthetic peptide, aa 44–64 from N-terminals of mouse	Original antibody, rabbit polyclonal (Kosaka et al., 2012; Takayama and Inoue, 2006)	1 μg/mL

CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase.
KCC2, potassium chloride co-transporter 2.

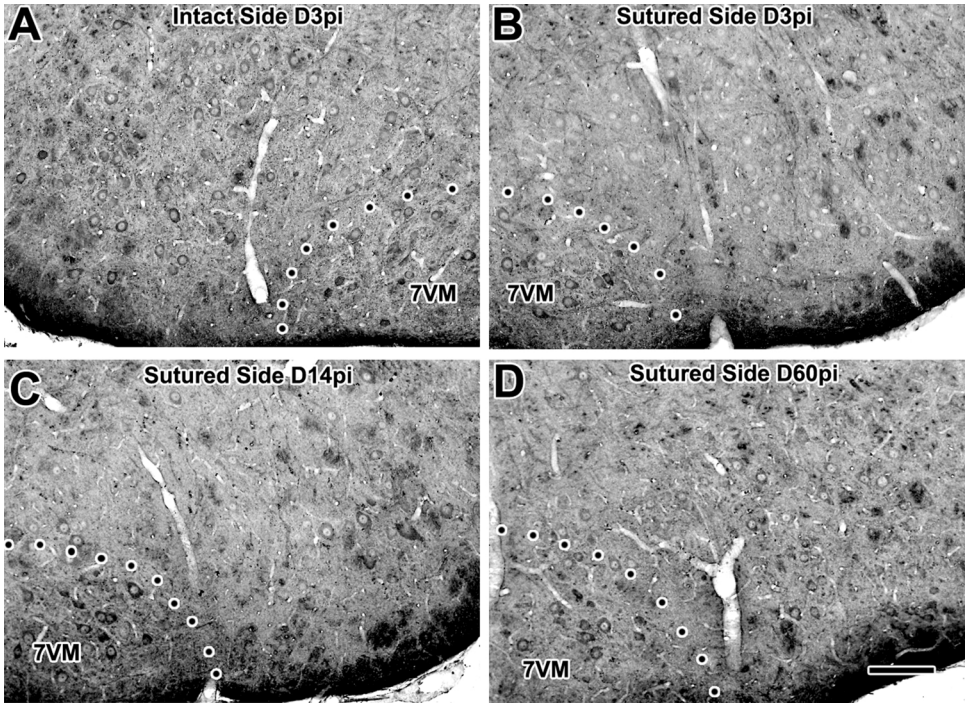

Fig. 1. Changes in immunohistochemical localization of choline acetyltransferase (ChAT) on the intact side (A) and sutured side (B–D) of the facial nucleus at 3 (A, B), 14 (C), and 60 (D) days after facial nerve operation. The majority of facial motor neurons (FMNs) were ChAT-positive on the intact side at day 3 (A). In contrast, ChAT immunolabeling disappeared from many of the motor neurons in the facial nuclei except for the ventromedial subnucleus (7VM) on the sutured side at day 3 (B) and day 14 (C). The ChAT-positive neurons were homogeneously detected in the facial nucleus at day 60 (D). D3pi: day 3 post-injury. Scale bar = 100 μm.

time course of these changes in expression and localization, and their relationship with functional recovery, have not been reported. In particular, it has not been clearly demonstrated whether these changes return to normal levels after re-innervation.

To address these points, we used the facial nerve transection and suturing model (Moran and Graeber, 2004) for the following reasons. First, the facial motor nucleus consists of six subnuclei and one accessory nucleus (Ashwell, 1982), which innervate their own facial muscles. In the present study, we transected the main trunk of the facial nerves except for the supraorbital nerve, which branches from the main trunk at the retroparotid region and originates from the ventromedial subnucleus (7VM). Therefore, 7VM can be used as an internal control. Second, facial motor nerve function can be evaluated using behavioral tests, such as blink reflex and vibrissae movement (Wang et al., 2012).

We have previously reported the changes in localization of GABAergic transmission-related molecules in the hypoglossal nucleus (Tatetsu et al., 2012), but did not investigate the relationship between changes in expression and the time course of neural functional recovery. In the present study, we focused on five molecules, including ChAT, CGRP, galanin, gephyrin, and KCC2, whose expressions are markedly altered in facial motor neurons (FMNs) after facial nerve injury. ChAT is a synthetic enzyme of the motor neuron neurotransmitter ACh, and one of the markers for motor neuron activity. CGRP and galanin are neuropeptides, expressed in FMNs, transiently increase in expression after axonal injury, and might be involved in extension of axons (Chen et al., 2010; Kim et al., 2016; Makwana et al., 2010). Both gephyrin and KCC2 play key roles in GABAergic transmission. Because gephyrin is an anchoring protein of both glycine (Gly) and GABA receptors (Fritschy et al., 2008; Kneussel and Betz, 2000; Tretter et al., 2008; Yu et al., 2007), it is considered a marker of inhibitory neurotransmitter receptors. KCC2 reduces $[Cl^-]_i$ and controls the action of GABA and Gly at the postsynaptic site (Ben-Ari, 2002; Owens and Kriegstein, 2002; Payne et al., 2003). Abundant expression of KCC2 shifts GABA action to inhibition, whereas disappearance of KCC2 shifts GABA action to excitation. We examined the time course of changes in the expression and localization of the above five molecules for about 2 months after facial nerve transection and suturing, almost the full time needed for the function to recover, and observed the changes in facial motor function.

2. Experimental procedure

2.1. Animals and surgery

We used 42 male C57BL/6J mice, 3 to 4 months old (SLC, Shizuoka, Japan), 7 mice for each time point. The animals were maintained at a