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Quantitative analysis of survival of hypoglossal neurons in neonatally nerve-injured rats: Correlation with milk intake

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ABSTRACT

Introduction: Tongue movement innervated by the hypoglossal (XII) nerve is essential for the survival of neonatal rats. The pups with bilateral XII nerve resection failed to suckle milk and did not survive, and the pups with unilateral XII nerve resection showed disturbed suckling capability and lower survival rates. The present study was performed to investigate the relation between neuronal population and milk intake of developing rats that had received various degrees of crush injuries to the unilateral XII nerve during the neonatal period.

Methods: The right XII nerve of postnatal day 1 (P1) pups was crushed and milk intake was estimated at 3 days and 6 days after the nerve injury. As nerve injury at the neonatal stage results in death of axotomized neurons, varying degrees of crushing was estimated by the number of survived motor neurons.

Results: In nerve-crushed rats, the populations of XII motor neurons and amounts of milk intake were reduced in a varied manner. Statistically significant positive correlations were observed between increasing XII neuron survival and increasing milk intake at 3 ($r = 0.62$) and 6 ($r = 0.71$) days after the nerve injury.

Conclusion: The results indicate that there is a strong relationship between the number of XII motor neurons and the amount of milk intake in neonatally XII nerve-injured rats.

1. Introduction

Newborn mammals cannot survive without effective suckling after birth. We previously reported the essential roles of the tongue in suckling in hypoglossal (XII) nerve-injured newborn rats.[1,2] We injured the XII nerve, which controls tongue movement, and revealed the effects of XII nerve injuries on milk intake by measuring the amounts of suckled milk. For suckling, pups need to make a semi-bowl shape of their tongues and alternately protrude and retract their tongues. We showed that postnatal day 1 (P1) pups with bilateral XII nerve resection failed to suckle milk and did not survive. Moreover, P1 pups with unilateral XII nerve resection also showed disturbed suckling capability and lower survival rates.

The present study was undertaken to investigate the relation between neuronal population and suckling capability of developing rats that had received various degrees of crush injuries to the unilateral XII nerve during the neonatal period.

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It is well known that neonatal neurons are vulnerable to axon injury, and peripheral nerve injury produces significant cell death in neonatal motor neurons.[3–6] Therefore, we estimated the degrees of nerve crushing by measuring the number of survived motor neurons in the XII nucleus.

2. Materials and methods

2.1. Animals

Newborn Wistar rats (Japan SLC Inc., Shizuoka, Japan) of both sexes were used in this study. Surgical procedures were performed after the induction of deep anaesthesia by hypothermia (−20 °C, 15 min). All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and the protocols were approved by our Institutional Animal Care and Use Committee. Every effort was made to minimize animal suffering and pain.

2.2. Unilateral injuries on the XII nerve

Under a surgical microscope, the right XII nerve of P1 pups ($n = 42$) was exposed and crushed at its main trunk in varying degrees with fine forceps. Great care was taken to expose the nerve itself and not to damage the surrounding tissues, including the tongue. For comparison, the remaining P1 pups were subjected to exposure of the right XII nerve and were used as controls ($n = 10$) or to total resection of their main trunk by a length of 1.5 mm ($n = 10$). Postoperatively, pups were housed with their dam in a single cage (26 cm × 42 cm × 18 cm) under standard laboratory conditions with a 12-h light/dark cycle and room temperature maintained at 22 °C. The pups were housed with the dam until P7. Some nerve-injured rats (crushed: $n = 5$; resected: $n = 6$) showed a continual decrease in body weight and died by P4.

2.3. Measurements of milk intake

At P4 and P7, milk intake was estimated in the same manner as described in our previous papers.[1,2,7] Briefly, we removed the siblings from the dam's cage and kept them apart from the dam in a box (13 cm × 25 cm × 9 cm) made of thick paper for 4 h. The temperature in the box was kept at 27 °C and the humidity was kept at 30% ± 2%. Just prior to lactation, the pups were induced to excrete urine adequately by pressing the abdomen and stimulating the external urethral orifice and anus with tissue paper, and then their body weights were measured. The pups were placed back in the dam's cage and she was allowed to lactate for 1 h. After 1 h of lactation, the pups' body weights were remeasured. The milk intake of each individual pup was determined as the difference in body weight just before and after lactation. The measurements were performed twice a day, and each pup's milk intake was defined as the average value.

2.4. Tissue preparation and quantification of the XII neurons

After measurements of milk intake at P7, rats were euthanatized with sodium pentobarbital (100 mg/kg, intraperitoneally) and perfused through the heart with 20 mL of 4% paraformaldehyde in 0.1 M phosphate buffer. The brainstem containing the XII nucleus was removed, postfixed overnight in the same fixative, dehydrated, and embedded in paraffin. The paraffin-embedded brainstem was cut into 4-μm sections in the coronal plane serially at 40-μm intervals with a microtome.

Table 1 – Amounts of milk intake and numbers of XII neurons.

Animal		Milk intake (g)		XII neurons		
		P4	P7	R	L	R/L (%)
Crush	1	0.32	0.53	782	808	96.8
	2	0.47	0.52	751	783	95.9
	3	0.39	0.43	662	691	95.8
	4	0.22	0.57	816	859	95.0
	5	0.27	0.49	689	725	95.0
	6	0.39	0.48	745	785	94.9
	7	0.29	0.47	746	791	94.3
	8	0.31	0.34	697	748	93.2
	9	0.41	0.50	777	836	93.0
	10	0.36	0.47	649	702	92.5
	11	0.30	0.46	627	678	92.5
	12	0.37	0.35	865	948	91.2
	13	0.32	0.59	833	919	90.6
	14	0.36	0.48	689	765	90.1
	15	0.24	0.42	743	825	90.1
	16	0.15	0.41	682	761	89.6
	17	0.28	0.46	704	806	87.3
	18	0.19	0.39	833	959	86.9
	19	0.34	0.57	807	939	85.9
	20	0.42	0.57	722	855	84.4
	21	0.33	0.59	598	718	83.3
	22	0.25	0.46	533	676	78.9
	23	0.26	0.36	493	637	77.4
	24	0.28	0.54	663	791	76.2
	25	0.29	0.49	564	793	71.1
	26	0.29	0.36	533	751	71.0
	27	0.25	0.44	645	911	70.8
	28	0.29	0.59	419	668	62.7
	29	0.30	0.42	410	662	61.9
	30	0.28	0.44	326	810	40.2
	31	0.28	0.28	237	627	37.8
	32	0.11	0.20	221	624	35.4
	33	0.18	0.27	233	694	33.6
	34	0.10	0.30	220	686	32.1
	35	0.30	0.37	267	844	31.6
	36	0.16	0.21	206	755	27.3
	37	0.07	0.27	159	651	24.4
Control	1	0.39	0.53	726	714	101.7
	2	0.12	0.53	816	808	101.0
	3	0.20	0.48	760	754	100.8
	4	0.42	0.68	839	839	100.0
	5	0.32	0.48	912	923	98.8
	6	0.38	0.36	753	766	98.3
	7	0.25	0.35	645	660	97.7
	8	0.38	0.53	687	706	97.3
	9	0.26	0.56	835	871	95.9
	10	0.27	0.51	799	866	92.3
Resection	1	0.11	0.08	140	896	15.6
	2	0.11	0.40	100	676	14.8
	3	0.12	0.32	89	682	13.0
	4	0.19	0.33	77	728	10.6