Short communication

Does aging alter the molecular substrate of ionotropic neurotransmitter receptors in the rostral ventral lateral medulla? - A short communication

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ABSTRACT

Aging alters sympathetic nervous system (SNS) regulation, although central mechanisms are not well understood. In young rats the rostral ventral lateral medulla (RVLM) is critically involved in central SNS regulation and RVLM neuronal activity is mediated by a balance of excitatory and inhibitory ionotropic neurotransmitters and receptors, providing the foundation for hypothesizing that with advanced age the molecular substrate of RVLM ionotropic receptors is characterized by upregulated excitatory and downregulated inhibitory receptor subunits. This hypothesis was tested by comparing the relative mRNA expression and protein concentration of RVLM excitatory (NMDA and AMPA) and inhibitory (GABA and glycinergic) ionotropic neurotransmitter receptor subunits in young and aged Fischer (F344) rats. Brains were removed from anesthetized rats and the RVLM-containing area was micropunched and extracted RNA and protein were subsequently used for TaqMan qRT-PCR gene expression and quantitative ELISA analyses. Bilateral chemical inactivation of RVLM neurons and peripheral ganglionic blockade on visceral sympathetic nerve discharge (SND) was determined in additional experiments. The relative gene expression of RVLM NMDA and AMPA glutamate-gated receptor subunits and protein concentration of select receptor subunits did not differ between young and aged rats, and there were no age-related differences in the expression of RVLM ionotropic $GABA_A$ and Gly receptors, or of protein concentration of select $GABA_A$ subunits. RVLM muscimol microinjections significantly reduced visceral SND by $70 \pm 2\%$ in aged F344 rats. Collectively these findings from this short communication support a functional role for the RVLM in regulation of sympathetic nerve outflow in aged rats, but provide no evidence for an ionotropic RVLM receptor-centric framework explaining age-associated changes in SNS regulation.

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1. Introduction

Physiological function is altered with advancing age, including changes in sympathetic nervous system (SNS) regulation (Seals and Esler, 2000; Kaye and Esler, 2005). Multiple lines of evidence suggest that advanced age is associated with an enhanced state of sympathetic activation. For example, total norepinephrine (NE) spillover, hepatomesenteric NE spillover, muscle sympathetic nerve discharge, and cardiac NE spillover are higher in older compared with young adults (Seals and Esler, 2000; Seals and Bell, 2004). Despite the documentation of marked age-related changes in SNS regulation, little information is available regarding the effect of advanced age on molecular mechanisms regulating central sympathetic neural circuits.

The rostral ventral lateral medulla (RVLM) plays a pivotal role in the regulation of central sympathetic nerve outflow (Horiuchi et al., 2004;

Kenney et al., 2011). The activity level of RVLM presympathetic neurons in young animals is mediated by a balance of excitatory and inhibitory states (Ito et al., 2000; Sved et al., 2002), due to glutamatergic excitation mediated by NMDA and AMPA ionotropic receptors, and GABAergic and glycinergic inhibition mediated primarily by $GABA_A$ and glycine (Gly) ionotropic receptors, respectively. Because functional interaction between RVLM excitatory and inhibitory ionotropic receptors play a critical role in determining the level of efferent sympathetic nerve outflow, it is possible to speculate that age-related modifications in RVLM ionotropic receptor may provide the molecular framework underlying changes in SNS regulation with advanced age. Consistent with this notion, previous studies have identified age-associated changes in mRNA and protein expression of ionotropic receptors in central nervous system areas associated with learning and memory (Magnusson et al., 2010; Cantanelli et al., 2014; Ruano et al., 2000; Rissman et al., 2006).

Given the pivotal role of the RVLM in SNS regulation in young rats, the functional balance of RVLM excitatory and inhibitory receptor systems, and the established age-associated changes in SNS regulation, we hypothesized that the molecular substrate of RVLM ionotropic receptors is altered with advanced age. To test this hypothesis we

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examined the relative gene expression of RVLM excitatory (NMDA and AMPA) and inhibitory (GABA and Gly) ionotropic neurotransmitter receptor subunits in young (3–4 months) and aged (22–24 months) Fischer (F344) rats. Because of the diverse array of RVLM ionotropic receptor subunits a comprehensive profiling of 20 RVLM ionotropic receptor subunits was completed (Table 1). We speculated that changes in the RVLM receptor substrate in aged rats would be represented by one of at least three potential profiles: selective upregulation of excitatory NMDA and AMPA ionotropic receptors, supporting a shift toward an active enhancement of neuronal excitation; selective downregulation of GABA and Gly ionotropic receptor subunits, supporting a role for disinhibition in mediating neuronal excitation; or a combined effect of enhanced excitatory and reduced inhibitory ionotropic receptors. The expected findings would be the first to demonstrate that advanced age modulates the balance of RVLM ionotropic receptor subunits, thereby providing a framework for the design of studies to modify age-related changes in central regulation of sympathetic nerve outflow by targeting specific receptor systems. In addition, chemical inactivation of RVLM neurons produced by bilateral Muscimol microinjections in aged rats was completed to assess RVLM functionality.

2. Methods

All procedures and protocols were approved by the Institutional Animal Care and Use Committees and were completed in accordance with the American Physiological Society's guidelines for research involving animals. Experiments were completed in young adult (3–4 months; n = 8, 336 ± 6 g) (age of sexual maturity is 1.5 months) and aged (22–24 months; n = 11, 442 ± 9 g) (median survival age is 24–26 months) male Fischer 344 rats (Charles River Laboratories, contracted with National Institute on Aging). F344 rats are a strain of rats that are provided by the National Institute on Aging for studies focused on aging research, and are widely used in this research domain (Mitchell et al., 2015). Many studies that have employed direct

sympathetic nerve recordings and central microinjections to determine the effects of advancing age on sympathetic nervous system regulation have utilized F344 rats as the preferred rodent model (Helwig et al., 2006; Kenney, 2010; Kenney et al., 2011).

2.1. Brain sectioning and micro-punching

Rats were deeply anesthetized with 5% isoflurane and sacrificed by decapitation. Immediately after sacrifice brains were removed and snap frozen in liquid nitrogen, then stored at −80 °C until further use. RVLM tissue samples were collected by the modified Palkovit's bilateral micropunch technique (Palkovits and Brownstein, 1983). Anatomical reference points were provided by the rat brain atlas of Paxinos and Watson (2014). The hindbrain was serial sectioned in the coronal plane from rostral to caudal using a cryostat. Two 200 μm rostral medullary area brain slices were collected (estimated distance from Bregma −12.2 mm). The RVLM containing area was identified using the lateral trigeminal tracts and the dorsal fourth ventricle as landmarks and micro-punched with a 0.5 mm diameter Harris micro-punch at −20 °C. Tissue collected from the micropunches was pooled in RNase free tubes and stored at −80 °C.

2.2. Central microinjections and sympathetic nerve recordings

RVLM microinjections and sympathetic nerve discharge (SND) recording protocols have been described previously (Kenney et al., 2011). Chemical inactivation of the RVLM was completed by bilateral muscimol microinjections (500 pmol, n = 3; microinjectate volume 100 nl for each injection).

2.3. RNA extraction

RNA from RVLM tissue punches was extracted using the RNeasy® Lipid Tissue mini kit (Qiagen, USA) and QIAzol® Lysis reagent. RNA was quantified using the Nanodrop D8000 (Thermo-Scientific, Wilmington, DE). The purity of RNA samples was assessed using the 260/280 absorbance ratio and samples demonstrating an absorbance ratio of 1.8–2.0 were used for cDNA preparation.

2.4. cDNA preparation and preamplification

Total RNA (100 ng) was converted to cDNA using a high capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA). Reverse transcription was performed using the BioRad iQ5TM thermal cycler under standard conditions, and cDNA samples were stored at −20 °C until further use. The number of reactions per sample was increased by preamplification which was completed using 10 ng of cDNA with 2× TaqMan® PreAmp master mix (Applied Biosystems, Foster City, CA). Following the preamplification phase, samples were immediately diluted with 1× TE buffer and stored at −20 °C until further use.

2.5. Quantitative real-time PCR (qRT-PCR)

The qRT-PCR was performed using StepOnePlus™ Mastercycler (Applied Biosystems, CA) and the Taqman™ assay mix (Life technologies, CA) for gene expression studies. Each of the 20 ionotropic receptor subunit genes was probed separately with GAPDH as the endogenous control. The qRT-PCR plates with 96 wells were run on a thermal cycler with standard experimental conditions.

2.6. Protein extraction and quantitative sandwich ELISA

Protein extraction was completed using the protein precipitation method from the organic phase remaining after RNA isolation (Simoes et al., 2013). Extracted protein samples were processed using standard procedures, aliquoted, and stored at −20 °C until further use. Protein

Table 1
(A) Ionotropic neurotransmitter receptor subunit genes included in the qRT-PCR gene expression analysis with respective average Ct values and (B) protein concentrations of select NMDA and GABA$_A$ receptor subunits in young and aged rat groups.

(A)	Ionotropic neurotransmitter receptor (mRNA)	Subunits	Young (Avg Ct value)	Aged (Avg Ct value)
	NMDA	Grin1	21.42	21.67
		Grin2a	24.35	24.33
		Grin2b	24.37	24.74
		Grin2c	23.24	23.62
	AMPA	Gria1	23.01	23.18
		Gria2	23.25	23.93
		Gria3	23.07	23.42
		Gria4	21.79	22.36
	GABA$_A$α	Gabra1	22.50	22.20
		Gabra2	23.12	22.44
		Gabra3	27.18	27.07
		Gabra4	26.50	26.83
		Gabra5	24.11	24.12
	GABA$_A$β	Gabrb1	24.59	24.04
		Gabrb2	22.78	23.24
		Gabrb3	22.55	23.15
	GABA$_A$γ	Gabrg1	22.62	23.05
		Gabrg2	21.75	22.08
	Gly	Glra1	21.89	22.27
		Glrab	21.14	21.39
(B)	Protein concentration	Subunits	Young	Aged
	NMDA	Grin1	13.77 ± 1.77 ng/ml	12.39 ± 2.11 ng/ml
		Grin2a	405.93 ± 5.23 pg/ml	406.15 ± 4.72 pg/ml
		Grin2c	154.39 ± 12.85 pg/ml	152.12 ± 8.97 pg/ml
	GABA$_A$	Gabra1	1.89 ± 0.14 μmol/l	2.02 ± 0.15 μmol/l
		Gabra2	329.06 ± 72.98 pg/ml	388.77 ± 78.35 pg/ml
		Gabrg2	1.32 ± 0.31 μmol/l	1.46 ± 0.25 μmol/l