DISTRIBUTION OF CALRETININ-IMMUNOREACTIVE INTERNEURONS IN DORSAL PART OF HIPPOCAMPUS IN ANIMAL MODEL OF DEPRESSION

Barbara NOWAK[1], Alicja BRANIEWSKA[1], Monika ZADROŻNA[1]

1. *Department of Pharmacobiology, Medical College, Jagiellonian University, 30-688 Cracow, Medyczna str. 9, Poland.*

Abstract

Calretinin-containing (CR+) GABAergic neurons in the dorsal part of hippocampal formation (HF), including the subiculum, Cornu Ammonis (CA1-4), and dentate gyrus (DG) were visualized with immunocytochemistry. General distribution of CR+ cells was similar in each studying group. Olfactory bulbectomy caused significant increase in CR+ density in stratum oriens of CA1 and stratum moleculare of suprapyramidal blade of DG (three times, $p<0.05$ and twice, $p=0.05$, respectively), and tendency to increase in majority of other sub-regions of HF. CR+ cells were generally resistant to administration of amitriptyline as an antidepressant following bulbectomy, although the tendency of increase in CR+ cell density can be observed in CA1. Our findings indicate CR+ neurons site-specific response to bulbectomy model of depression. This could involve the trisynaptic pathway and temporo-ammonic tract by controlling other interneurons terminating on different compartments of principal cells.

Key words: bulbectomy, calretinin, hippocampus, interneuron

Corresponding author: barbara.anna.nowak@uj.edu.pl

Introduction

Gamma-aminobutyric acid (GABA-ergic) inhibition has a prominent role in the brain control of stress, the most important vulnerability factor in mood disorders. Reorganization of hippocampal GABA interneuron microcircuits are considered to play a substantial or even causal role in major depressive disorders [4,5,13], in schizophrenia [23], in epileptogenesis [7,11,14] and contributes to the development of autism [12]. In the majority of cases non-principal neurons seem to be resistant or are present in large numbers in the epileptic human hippocampus [2,17]. Although in contradiction, the selective deficit of some interneuron populations has also been reported [8,15].

Distinct subpopulation of GABAergic inhibiting cells containing calretinin-binding protein (CR) was proved to be vulnerable to ischemic, and epileptic injury both in animal models [1,14,22,19], and in humans [20,15,18]. In this study, we aimed to shed light on the distribution of hippocampal CR+ neurons following bulbectomy as the animal model of major depressive disorders. This part of investigation refers to dorsal part of hippocampal formation.

Materials and methods

The hippocampi were excised from four groups of rat: control sham-operated (C), bulbectomized (B), and after four-week amitriptyline treatment following bulbectomy (BA), and after four-week amitriptyline administration following sham operation (A) rat (n = 6 for each group). After removal tissues were immediately immersed into a fixative containing 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) and placed in paraffin. Nine micrometer slices were cut for immunostaining according to standard LAB-SA method (LAB-SA Detection System, ZYMED, USA). Sections were dewaxed and rehydrated with standard procedures. To retrieve antigen, slides were heated from 92 to 96ºC in a 10 mM citrate buffer (pH = 6.0) for 20 min. Next, they were quenched sequentially in 1% H2O2 and methanol for 30 min, blocked with

normal serum (ZYMED Labs, USA) and incubat-incubated in a moist chamber at 4ºC overnight with primary antibody (polyclonal anti-calretinin, Millipore, 1:200). A several washes of sections in PBS were followed by incubation with the appropriate biotinylated secondary antibodies (ZYMED Labs, USA) for 10 min at room temperature, then followed by the streptavidin-peroxidase complex (ZYMED Labs, USA) for 10 min at room temperature. The binding of primary antibody was visualized using diaminobenzidine (Invitrogen Ltd., UK) for 8 min. After washing with distilled water (some sections were counterstained with a Nissl substance) slides were dehydrated in ethanol and xylene and mounted in the DPX medium (Fluka). Immunostaining controls: no labeling was detected when primary antibodies were omitted. All samples were examined at the light microscopic level. To obtain data on the density of calretinin-positive cells, three to four representative sections of the hippocampi (from 2.0 mm to 3.0 mm level from bregma, according to Paxinos and Watson 2005, Witter 2011) were drawn by camera lucida to delineate standard regions of hippocampus: subiculum (distinguishing strata oriens, pyramidale and moleculare), CA1 (strata oriens, pyramidale, radiatum and lacunosum-moleculare), CA3 (strata oriens, pyramidale and radiatum+lacunosum-moleculare), CA4, dentate gyrus – suprapyramidal blade (strata granulosum, moleculare and subrgranular zone), and the same three layers for dentate gyrus – infrapyramidal blade, and hilus. The surface area of each region was measured by the CellD software. The quantity of CR+ cells was determined per unit area (mm2). Data were evaluated by the Statistica 10.0 software. Kruskal Walis one-way analysis of variance and Dunn's multiple comparison post-hoc tests were applied.

Results and Discussion

CR immunoreactivity was present in non-principal cells in all sub-regions of dorsal HF (Fig.1) and the distribution of CR+ cells was generally similar in each included group. The highest density had interspersed cells in the strata of principal cells: stratum pyramidale of the CA and granule cell layer of DG. Immunostaining marked the soma, and the proximal and distal dendrites of interneurons.

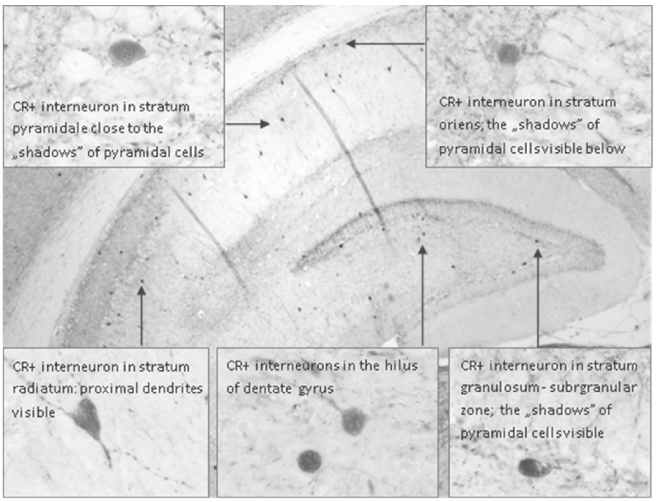

Fig. 1 The representative microphotographs of calretinin immunoreactivity in dorsal hippocampus (original magnification in main photograph, x 40; in boxes, x 1000).

The numerical density and numerical indexes of CR+ neurons on the background of their detailed distribution is summarized in Table 1 and 2. In most sub-regions of HF of bulbectomized rats, the preservation or even an increase of the number of CR+ cells in comparison to control was observed (Tables.1-2). The largest density of immunoreactive cell was found in the strata pyramidale (for CA3: 9.98 cell/mm2 in control HF and 11.20 cell/mm2 in bullbectomized HF) and radiatum of CA (Fig. 4), in the hilus (6.61 cell/mm2 and 11.38 cell/mm2 respectively), and in the subgranular zone of the suprapyramidal blade of the DG (8.19 cell/mm2 and 8.34 cell/mm2 respectively; considering the density specified for each layer, keep in mind that this amount is normalized to the surface of

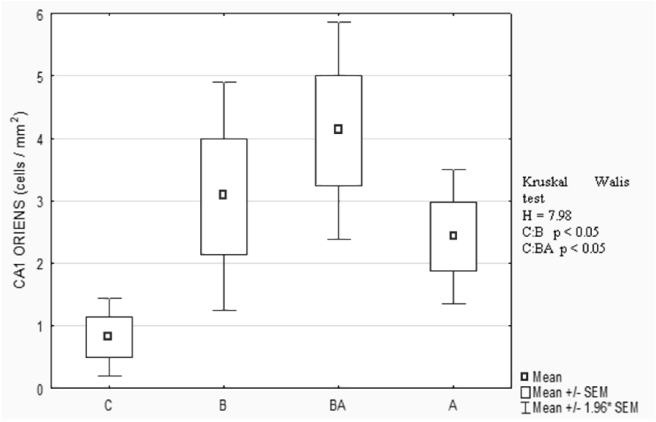

Fig. 2 The stratum oriens of CA1 sub-region of dorsal hippocampus; the density of calretinin-containing cells in control samples (C), bulbectomized (B), and amitriptyline treated following bulbectomy (BA), and following sham-operating (A) samples.

the sub-region, to avoid inaccurate measurement error). Although the proportionally lesser number of CR+ cells was visible in stratum oriens of CA1: 3.07 cell/mm2 and stratum moleculare of DG: 5.05 cell/mm2 of bulbectomized rats, these were the sites of most striking up-regulation CR+ cells ($p<0.05$, Fig.1, 2) versus control tissue.

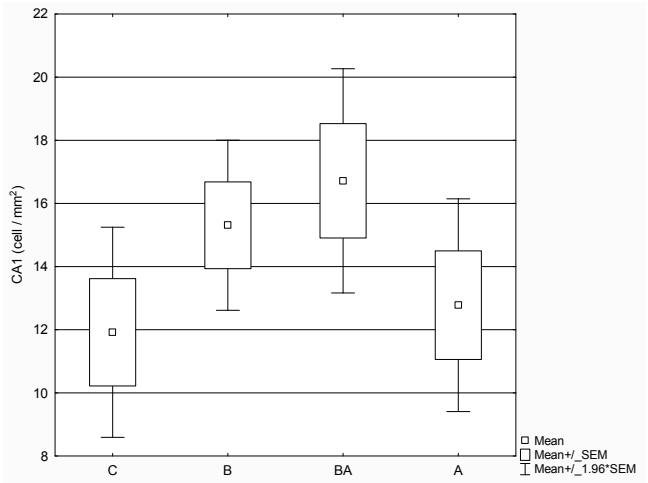

Fig. 3 The CA1 sub-region of dorsal hippocampus; the density of calretinin-containing cells in control samples (C), bulbectomized (B), and amitriptyline treated following bulbectomy (BA), and following sham-operating (A) samples.

Substantial changes in stratum oriens in GABAergic dysfunction were proved also at the level of gene regulation of specific interneurons proteins [4,5,6]. Antidepressant administration tends to further up-regulation of this population of GABAergic neurons (BA versus B, and A versus C; Fig. 4, Tables. 1, 2). Positive correlations in density or CR+ cells between consecutive sub-regions along the trisynaptic entorhino–hippocamal projection were identified (Fig. 5); i.e. DG:CA4, CA4:CA3, CA3:CA1, CA1:subiculum, which confirms the consistency of observed changes.

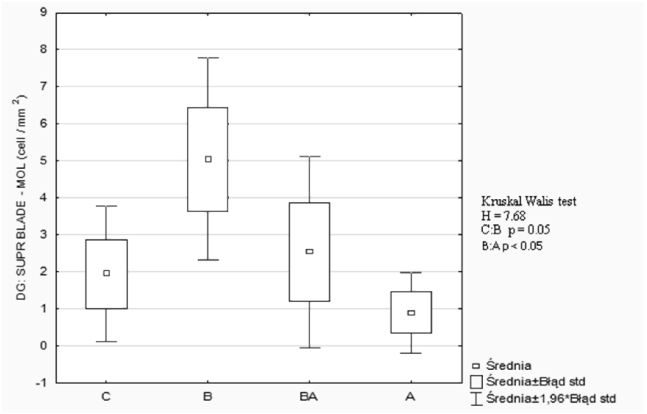

Fig. 4 The stratum granulosum – subgranular zone of DG sub-region of dorsal hippocampus; the density of calretinin-containing cells in control samples (C), bulbectomized (B), and amitriptyline treated following bulbectomy (BA), and following sham-operating (A) samples.

Calretinin-containing neurons are high selective inhibitory cells in the rat hippocampus targeting dendritic inhibitory cells (most of all calbindin containing neurons) and other sisterly calretinin-containing interneurons, and forming dendro-dendritic massive contacts with each other [20]. The cells are responsible for the synchronization of dendritic inhibitory cells, which has a prominent role for an efficient control of principal cells. Dendritic inhibitory neurons can prevent the generation of dendritic calcium spikes and restrict synaptic plasticity. Thus calretinin-containing neurons involve as a "disinhibitory" neurons [10,20,21].

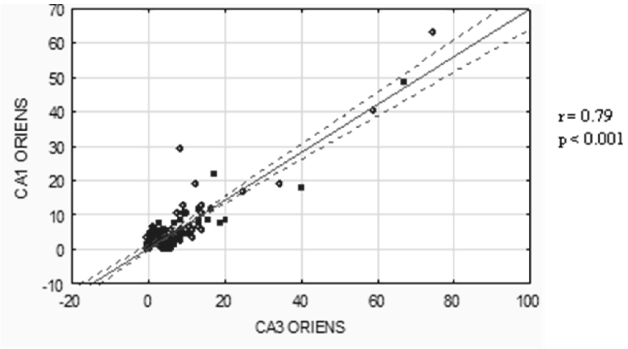

Fig. 5 Positive correlation in density or CR+ cells between consecutive CA1: CA3 regions (stratum oriens) in run of trisynaptic pathway of entorhinal cortex – hippocampus projection.

The question which emerges is: which kind of neuromodulation factor is likely to make the calretinin-immunoreactive cells resistant or more numerous following bulbectomy in a site-dependent manner? Although it was not confirmed by the statistic tests, which problem probably arises from a small amount of cases, the cells display at last the tendency to up-regulation in density in almost all sub-regions. It is noteworthy that the location of the significantly growing cell density overlaps with two main entorhino-hippocampal projections, namely classic perforant pathway crossing the hippocampal fissure to continue into stratum moleculare of dentate gurus, and the another temporo-ammonic tract. Axons in the temporo-ammonic tract travel through stratum oriens of CA, and eventually traverse into the most superficial layers. The number of entorhinal fibers that projects the temporo-ammonic way increase precisely in dorsal level of hippocampal formation. This should be examined in comparison in ventral part of hippocampus, as these two pools of hippokampi are regarded as two distinct units now [9].

Conlusion

We state that olfactory bulbectomy caused significant increase in calretinin containing cells density in stratum oriens of CA1 and stratum moleculare of suprapyramidal blade of DG of dorsal part of hippocamus. This site-specific reorganization overlaps spatially with two "gates" for entorhino-hippocampal projections, perforant pathway and temporo-ammonic tract. Antidepressant treatment tends to further up-regulation of this population of GABAergic neurons.

Future studies should explore the relationship between other supopulations of interneurons co-creating GABAergic system and should clarify the potential mechanisms of the reorganization of calretinin containining neurons in bulbectomy modeled depression.

Table 1 Data on calretinin-positive cell density distribution in the Cornu Ammonis of dorsal rat hippocampus (cells/ mm2) for subiculum and CA1 – CA4 sub-regions; OR, stratum oriens; PYR, stratum pyramidale; MOL, stratum moleculare; RAD, stratum radiatum; LM, stratum lacunosum-moleculare), R+LM, strata radiatum+lacunosum-moleculare; SPYR, IPYR, area located over and below (supra- and infra-) dense group of cells; for control (C), bulbectomized (B), and amitriptyline treated (BA and A) grups.

	SUBICULUM			**CA1**				**CA3**			**CA4**		
	OR	PYR	MOL	OR	PYR	RAD	LM	OR	PYR	R+LM	SPYR	PYR	IPYR
						C n=6							
_ X	**2.81**	**2.97**	**2.61**	**1.26**	**6.69**	**2.56**	**1.41**	**1.84**	**9.98**	**3.94**	**3.53**	**6.35**	**5.90**
range	0.00– 8.87	0.99– 5.92	1.18– 4.51	0.00– 2.01	2.31– 10.89	0.99– 3.99	0.00– 3.63	0.00– 5.42-	3.36– 15.17	3.25– 6,53	0.00– 7.37	0.00– 11.73	0.00– 17.12
SEM	1.28	0.74	0.54	0.31	1.28	0.42	0.54	0.82	1.90	0.31	1.51	1.86	2.7
						B n=6							
_ X	**2.35**	**4.46**	**2.08**	**3.07**	**8.11**	**3.11**	**1.01**	**2.09**	**11.20**	**5.79**	**2.17**	**7.12**	**7.23**
range	0.00– 5.06	1.90– 6.52	0.00– 4.68	0.75– 6.36	5.12– 10.44	1.21– 5.22	0.00– 1.91	0.00– 4.56	8.39– 19.96	3.69– 8.65	0.00– 5.65	2.16– 9.65	0.00– 16.39
SEM	0.69	0.69	0.83	0.93	0.93	0.55	0.35	0.72	1.76	0.87	0.85	1.13	2.24
						BA n=6							
_ X	**1.94**	**3.33**	**3.63**	**4.13**	**9.05**	**2.58**	**0.95**	**1.21**	**10.68**	**5.68**	**2.67**	**8.29**	**4.35**
range	0.00– 7.04	0.00– 7.11	0.00– 9.38	0.00– 7.37	3.18– 12.52	0.77– 5.44	0.00– 2.48	0.00– 2.74	6.39– 14.01	2.74– 8.57	0.00– 5.33	0.00– 19.33	0.00– 10.52
SEM	0.92	1.17	1.24	0.88	1.4	0.58	0.32	0.32	1.21	0.89	0.81	2.25	1.45
						A n=6							
_ X	**1.34**	**3.20**	**2.75**	**2.40**	**5.97**	**2.38**	**1.99**	**1.94**	**11.99**	**4.93**	**1.32**	**4.90**	**5.81**
range	0.00– 5.41	0.00– 8.17	0.00– 5.41	0.00– 5.05	2.41– 11.77	0.60– 4.15	0.00– 3.97	0.00– 4.39	6.97– 18.42	2.18– 7.52	0.00– 4.05	0.00– 10.47	0.00– 14.47
SEM	0.56	1.00	0.49	0.55	0.96	0.42	0.50	0.55	1.28	0.60	0.54	1.15	1.51

Table 2 Data on calretinin-positive cell density distribution in the dentate gyrus of dorsal rat hippocampus (cells/ mm2) for hilus, suprapyramidal and infrapyramidal blades; MOL, stratum moleculare; GRAN, stratum granulosum, SUBGR, subrgranular zone; groups C, B, BA, and A as in Table.1.

	HILUS	**SUPRAPYRAMIDAL BLADE**			**INFRAPYRAMIDAL BLADE**		
		MOL	GRAN	SUBGR	MOL	GRAN	SUBGR
				C n=6			
_ X	**6.61**	**1.95**	**0.81**	**8.19**	**0.29**	**0.86**	**2.07**
Range	0.00 - 14.2	0.00 - 5.23	0.00 - 2.84	1.64 - 10.5	0.00 - 2.06	0.00 - 3.63	0.00 - 10.9
SEM	2.90	0.97	0.48	1.35	0.34	0.6	1.78
				B n=6			
_ X	**11.38**	**5.05**	**1.33**	**8.34**	**2.01**	**0.00**	**3.16**
Range	0.00 - 19.3	2.28 - 11.3	0.00 - 3.07	0.00 - 16.1	0.00 - 4.26	_	0.00 - 10.4
SEM	5.20	1.39	0.60	2.44	0.72		1.69
				BA n=6			
_ X	**6.45**	**2.54**	**1.31**	**7.74**	**3.81**	**0.00**	**2.59**
Range	0.00 - 14.4	0.00 - 9.18	0.00 - 3.02	0.00 - 19.1	0.00 - 8.92	-	0.00 - 8.82
SEM	3.89	1,32	0.39	2.16	1.40		1.43
				A n=6			
_ X	**7.50**	**0.90**	**1.11**	**4.51**	**1.05**	**0.00**	**3.70**
Range	0.00 - 23.7	0.00 - 4.96	0.00 - 5.52	1.29 - 23.9	0.00 - 2.54	-	0.00 - 9.99
SEM	3.17	0.55	0.60	2.31	0.41		1.16

Resumo

En tiu ĉi artikolo oni priskribas elektitajn kulturojn de GABA-ergiaj ĉeloj en hipokampoj de ratoj, kiuj havis forigitajn flarsentajn organojn kaj efikon de amitryptylino. Forigo de flarsentaj organoj estas la modelo de neŭrodegeneraciaj malsanoj de bestoj, en ĝi depresio. En multaj esploroj oni konstatis malakcelan efikon de GABA-ergia sistemo en afektiva dupolusa malsano kaj en depresio.

Niaj esploroj demonstras, ke en aplikanta modelo de depresio ŝanĝiĝas dispartigo de kalretininaj ĉeloj kaj ĝia denseco pligrandiĝas laŭ modelo precize kunligita kun loko en hipokampo (diversaj partoj de hipokampo). En multaj regionoj ankaŭ amitriptilino uzata kiel antidepresilo (pri konata neŭrogeneza efiko) kaŭzas pligrandigon de de denseco de kalretininaj ĉeloj.

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