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Interaction effects between vascular factors, age-associated nigrostriatal dopaminergic losses and neurodegenerations: a multisystem model of motor impairments in vascular parkinsonism

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Abstract

Vascular parkinsonism (VP) is a heterogeneous entity. VP is likely not the exclusive result of disruption of motor pathways by vascular lesions. Older age is a significant risk factor for VP and nigrostriatal losses of normal aging are likely to interact with vascular factors in the etiopathogenesis of parkinsonism. Furthermore, age-related prodromal neurodegenerations such as incidental Lewy bodies and amyloidopathy may also expand the spectrum of overlapping pathologies seen in VP. A multi-system model may explain motor parkinsonism in elderly subjects with VP where motor symptoms arise from a combination of vascular, aging and prodromal neurodegenerative etiologies.

Keywords: Aging, dopamine, amyloid, Lewy body, Parkinson disease, vascular parkinsonism

Introduction

Pure or non-Lewy body vascular parkinsonism (VP) can occur in at least two forms. First, the more rare and typically acute form of pure VP (primary VP or PVP) can manifest itself when a clear ischemic or hemorrhagic stroke occurs in the substantia nigra or nigrostriatal pathway that leads to a distinct presynaptic dopaminergic lesion (with or without post-synaptic dopamine receptor losses). Pure VP parkinsonism is only proven when there is a clear prior ischemic or hemorrhagic event that occurs in the substantia nigra or nigrostriatal pathway that leads to presynaptic dopamine transporter deficiency [1]. Second, a more common form may occur when typically widespread extra-nigrostriatal white or gray matter vascular lesions associate with motor parkinsonism (secondary VP or SVP). Although there may not be a distinct focal nigrostriatal deficit in SVP, it is assumed that motor parkinsonism occurs when vascular lesions disrupt connectivity in widespread neural systems underlying bipedal stance and gait that include cortico-striatal-thalamo-cortical loops, interhemispheric fibers, striatothalamic-brainstem systems, and proper processing of multi-modal sensory information [2]. The second type of VP develops more insidiously but it is debatable how primary the role of vascular lesions is in its etiopathogenesis. It is evidence that aging appears to play a key role in the emergence of motor symptoms in this group of patients with more widespread cerebrovascular disease [2].

Methods

Review of recent in vivo imaging PET, SPECT and MRI papers addressing age-associated nigrostriatal losses and prodromal neurodegenerations and possible interactive effects between cerebrovascular lesions and neurodegenerative changes.

Results

The threshold for the emergence of motor symptoms in Lewy body parkinsonism (LBP) is at least 50% loss of dopaminergic nerve terminals in the posterior putamen [3]. Normal aging is also associated with loss of nigrostriatal nerve terminals and can be as high as 7-8% loss per decade in adult life [4, 5]. Therefore, a septuagenarian may incur a loss of 40% or more of nigrostriatal nerve terminals, which is below but not far from the LBP motor threshold. Any slight burden of vascular disease in such individual may tip them toward the

manifestation of subtle motor impairments. For example, Louis and colleagues described mild parkinsonian features in 16.7% of community-dwelling elderly associated significantly with vascular burden [6]. Therefore, it is conceivable that cerebral vascular lesions lower the threshold for developing parkinsonian symptoms in the elderly with age-associated nigrostriatal dopaminergic losses. The co-occurrence of LBP and vascular disease is also common resulting in mixed pathologies. For example, up to half of patients with autopsy-confirmed Lewy body confirmed Parkinson disease (PD) were found to have vascular lesions [7-9]. Fig. 1 illustrates the overlap between vascular disease, age-related nigrostriatal dopaminergic losses and Lewy body parkinsonism.

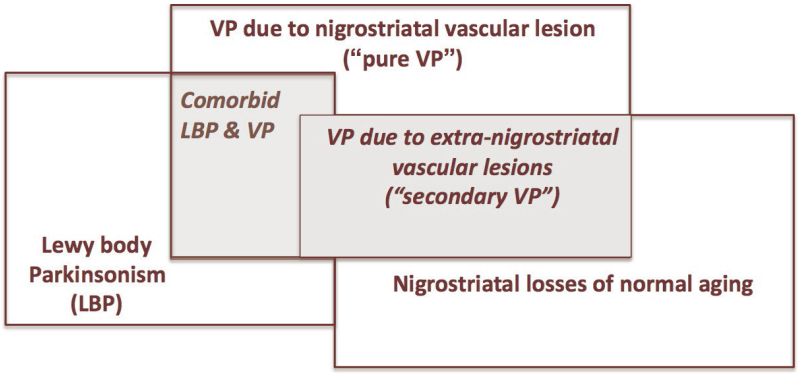

***Fig. 1**: Venn diagram showing overlap between vascular disease, age-related nigrostriatal dopaminergic losses and Lewy body parkinsonism.*

The presence of vascular lesions in PD have been selectively associated with abnormal gait and balance disturbances after accounting for the loss of nigrostriatal nerve terminals [10], and may explain why so-called "lower body parkinsonism" or cerebrovascular gait disorder is a common clinical presentation of VP [11].

Incidental Lewy bodies can be seen in 13.4% and β-amyloid plaques in up to 44% of cognitively normal elderly [12, 13]. Therefore, the combined presence of vascular disease and Lewy or Alzheimer proteinopathies will be common in elderly subjects with VP. There is evidence of cumulative or interactive effects of amyloidopathy in patients with subcortical vascular dementia. A recent β-amyloid brain PET and MRI study showed that amyloid plaque burden contributed to longitudinal cognitive decline [14]. Interestingly, amyloidopathy was the strongest poor prognostic factor in this vascular dementia study. In this respect, it is noteworthy that age-related amyloidopathy may also aggravate axial motor burden, at least in PD [15]. Cerebral small vessel disease, especially when located at the frontal ventricular horns, may also result in cortical cholinergic deafferentation based on the so-called "fiber disruption" hypothesis and is associated with cognitive changes in otherwise normal elderly subjects [16]. Cortical cholinergic denervation has also been implicated in mobility impairments with slower gait speed in PD [17]. There is also evidence of a dose-response relationship between freezing of gait in PD and the presence of cortical cholinergic denervation and amyloidopathy where freezing of gait is more common when both extra-nigral degenerations are present compared to either one alone [18]. This dose-response relationship points to interactive effects between these two extra-nigral conditions. This observation is of particular relevance to VP as freezing of gait can also be observed in this disorder.

Discussion: A multisystem model of mobility impairments in VP

VP is likely a combination of not only disruption of post-striatal motor pathways by vascular lesions but also dysfunction of presynaptic nigrostriatal nerve terminals. This may result from different etiologies. First, in the absence of Lewy body pathology, the integrity of nigrostriatal nerve terminals may be compromised by the effects of normal aging [4, 5]. Second, t is also possible that vascular lesions could accelerate nigral degeneration since multi-ischemic insults can induce excitotoxicity or cerebral hypoperfusion and increase the vulnerability of the nigrostriatal neurons [19, 20]. These factors underscore the heterogeneous nature of VP where different systems or mechanisms may interact with each other in the symptomatic presentation of motor parkinsonism.

Geriatric mobility problems are now being recognized as the result of deterioration of multiple physiological systems, including vascular or metabolic systems [21]. In this conceptual model, a relatively isolated impairment of a single system may not manifest clinical impairments because of adaptive plasticity in remaining intact systems. Once multisystem impairments occur, the surviving components of these systems cannot adapt further and clinical mobility problems become manifest, often in nonlinear fashion as critical thresholds are exceeded (Figure 2) [21, 22].

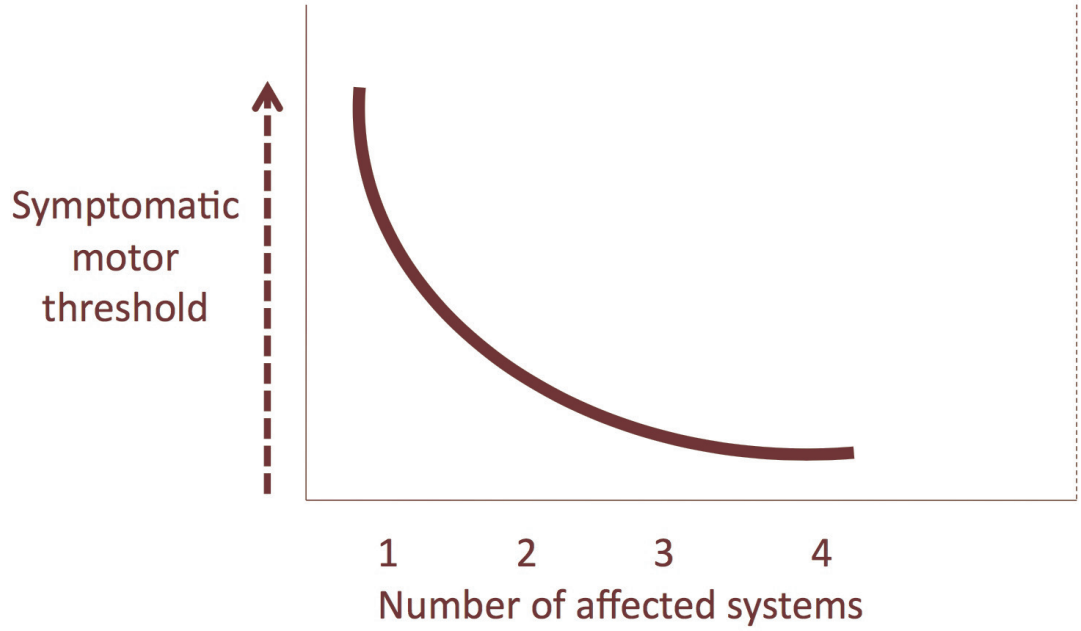

***Figure 2**: Multisystem degeneration in VP may resemble multisystem deteriorations underlying mobility problems and frailty in the elderly [21, 22]. Relatively isolated impairment of a single system may not manifest clinical impairments because of adaptive plasticity in remaining intact systems. Once multisystem impairments occur, the surviving components of these systems cannot adapt further and clinical morbidity becomes manifest, often in nonlinear fashion as critical thresholds are exceeded.*

Increased brain vascular lesion burden in the presence of degraded striatal function due to age-associated nigrostriatal losses or other causes likely represents a critical interactive mechanism exceeding the threshold underlying motor parkinsonism in SVP. The comorbid presence of cerebrovascular disease and LBP would follow the same multisystem model of motor parkinsonism but in a more pronounced and explicit form due to significant lowering of the symptomatic motor threshold in the setting of an even more degraded striatal capacity and other neurodegenerations in LBP. This multisystem degeneration model would explain also incremental motor effects of other conditions beyond pure vascular lesions in SVP, such as metabolic disorders like diabetes mellitus [23]. The presence of heterogeneous vascular, age-related and (prodromal) proteinopathies can explain a wide clinical spectrum of not only motor but also non-motor, including mood and cognitive, changes in VP [11].

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Endothelial dysfunction in patients with leukoaraiosis

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Abstract

Background: The pathophysiology of leukoaraiosis (LA) is incompletely understood. Theories of ischaemic genesis and a leaky blood-brain barrier are contradictory yet could share a common denominator – endothelial dysfunction, which has not been studied thoroughly in LA. **Methods:** Thirty patients with LA and 30 gender- and age-matched controls without LA were recruited. The vascular risk factors (VRF) were identical in both groups. Cerebral endothelial function was determined by cerebrovascular reactivity to L-arginine (CVR). Systemic endothelial function was determined by flow-mediated dilatation (FMD) of the brachial artery after hyperaemia. **Results:** both CVR (9.6 ± 3.2 % vs. 15.8 ± 6.1 %, $p<0.001$) and FMD (4.8 ± 3.1% vs. 7.4 ± 3.8 %, $p=0.004$) were markedly and significantly decreased in LA patients compared to controls. In LA patients, CVR and FMD significantly positively correlated ($b=0.192$, 95% CI=0.031-0.354, $p=0.02$). **Conclusions:** patients with LA seem to have a significant impairment of both cerebral and systemic endothelial function that is larger than could be expected based on present VRF. This may reflect "intrinsic" generalised endothelial dysfunction, which could be the initial, primary event in LA pathophysiology.

Keywords: cerebrovascular reactivity, endothelial dysfunction, flow-mediated dilatation, L-arginine, leukoaraiosis.

Introduction

It is well known that leukoaraiosis (LA) is associated with cognitive decline and a higher risk for stroke and death [1]. Despite this, LA pathogenesis is incompletely understood. The concepts of ischemic genesis [2] and a defective blood-brain barrier [3, 4] seem to oppose each other. Nevertheless, the cerebral endothelial dysfunction might explain both; however, recent studies have not clearly resolved the issue [5]. Thus, in the present studies, the harmful effects of vascular risk factors (VRF) on endothelial function have not been delineated from possible primary endothelial dysfunction in patients with LA. The cerebrovascular reactivity to L-arginine (CVR) is reported to be a reliable marker for cerebral endothelial function [6, 7]. Nevertheless, the method has not yet been used to determine cerebral endothelial function in LA. On the other hand, the flow-mediated dilatation (FMD) is a widely used method for the evaluation of systemic endothelial function [8], which has already been applied in LA. Contrary to previous studies, we aimed at estimating both systemic and cerebral endothelial function in patients with LA. The aim of this study was to investigate the CVR and FMD in patients with LA. In order to determine the role of primary endothelial impairment in LA and to eliminate the confounding effects of VRF on LA, we compared CVR and FMD in LA patients with that of patients with similar VRF without LA.

Materials and Methods

The study was performed in two groups of patients sharing identical VRF. The inclusion age was between 45 and 65 years. The LA group consisted of patients with visible LA in brain magnetic resonance imaging. The control group consisted of patients who did not have any radiological signs of LA. The VRF were evaluated based on a standardized interview, encompassing patient history, neurological examination, body mass index determination, laboratory tests and electrocardiography. Patients with diabetes were not included. Subjects with a known source of cardiogenic embolism were excluded. Leukoaraiosis was classified into four subgroups according to the Fazekas score [20]. Flow-mediated dilatation of the right brachial artery was studied [21]. A

hyperaemic flow increase was induced by inflation of a blood pressure cuff placed around the forearm. The FMD was expressed as the percentage change in the artery diameter after reactive hyperaemia relative to the baseline scan. The cerebrovascular reactivity to L-arginine (CVR) was determined. The mean arterial velocity (v_m) was recorded bilaterally in the trunks of both middle cerebral arteries through the temporal acoustic windows. Throughout the procedure, the mean arterial blood pressure, the partial pressure of exhaled CO_2 and the heart rate were measured continuously. The v_m was determined during the 10 min rest interval and the 10 min interval following the cessation of L-arginine infusion. Linear regression was used to explore the correlation between CVR and FMD in patients with LA. A 95% confidence interval (CI) was used. For any statistical test used in the study, $p \leq 0.050$ was regarded as statistically significant.

Results

The baseline characteristics of patients in both groups are shown in Tab. 1.

Tab. 1. Baseline characteristics and vascular risk factors in patients with leukoaraiosis (LA) and control group

	LA group (n=30)	**Control group (n=30)**	**p-value**
Age	58 ± 7 years	55 ± 6 years	0.08
Men	17 (56.7%)	20 (66.7%)	0.60
Current smoker	5 (16.7%)	5 (16.7%)	0.64
Hypertension	19 (63.3%)	16 (53.3%)	0.60
Dyslipidemia	13 (43.3%)	16 (53.3%)	0.61
Minor stroke/TIA	13 (43.3%)	0 (0.0%)	<0.01
Lacunar infarction	9 (30.0%)	0 (0.0%)	<0.01
Major ischemic infarction	1 (3.3%)	1 (3.3%)	0.45
Focal neurological deficits	13 (43.3%)	4 (13.3%)	0.04
Gait disturbance	12 (40.0%)	2 (6.7%)	0.01
Carotid plaques	16 (53.3%)	10 (33.3%)	0.19
Ischemic heart disease	3 (10.0%)	2 (6.7%)	0.60
Antihypertensive therapy	19 (63.3%)	15 (50.0%)	0.44
Statin therapy	14 (46.7%)	9 (30.0%)	0.29
Antiplatelet therapy	20 (66.7%)	7 (23.3%)	<0.01
Systolic blood pressure	146 ± 21 mmHg	140 ± 15 mmHg	0.25
Diastolic blood pressure	89 ± 10 mmHg	84 ± 9 mmHg	0.07
Body mass index	28 ± 4 kg/m^2	29 ± 5 kg/m^2	0.37
Intima-media thickness	0.74 ± 0.11 mm	0.72 ± 0.11 mm	0.52

The FMD was markedly and significantly diminished in patients with LA compared to patients with similar VRF without LA (4.8 ± 3.1% vs. 7.4 ± 3.8 %, p=0.004; Fig. 1A). The FMD was markedly and significantly diminished in the subgroup Fazekas 3 compared to the subgroup Fazekas 1 (3.0 ± 2.2 % vs. 6.4 ± 3.1%, p=0.01; Fig. 1B).

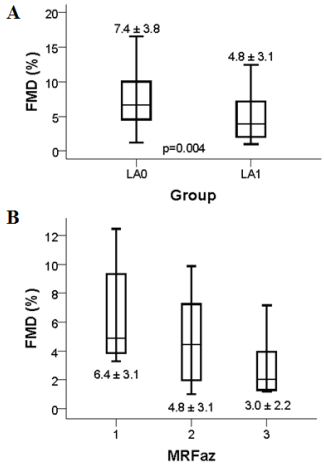

Fig. 1. Flow-mediated dilatation (FMD) distribution in control group and leukoaraiosis (LA) group (A) and in the subgroups of LA (B)

The CVR was markedly and significantly lower in patients with LA compared to patients with similar VRF without LA (9.6 ± 3.2 % vs. 15.8 ± 6.1 %, p<0.001; Fig. 2A). Patients with Fazekas 3 score had significantly and markedly lower CVR compared to patients with Fazekas 1 score (7.4 ± 3.1 % vs. 12.2 ± 2.6 %, p=0.001; Fig. 2B) and Fazekas 2 score as well (9.2 ± 1.9 %, p=0.008; Fig. 2B).

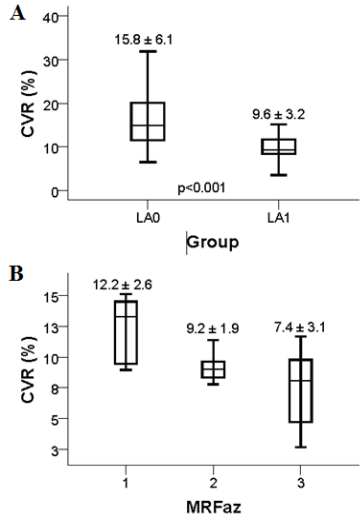

Fig. 2. Cerebrovascular reactivity to L-arginine (CVR) distribution in control group and leukoaraiosis (LA) group (A) and in the subgroups of LA (B). SD indicates standard deviation.

Linear regression showed a significant positive correlation between CVR and FMD in patients with LA (b=0.192, 95% CI=0.031-0.354, p=0.02; Figure 3).

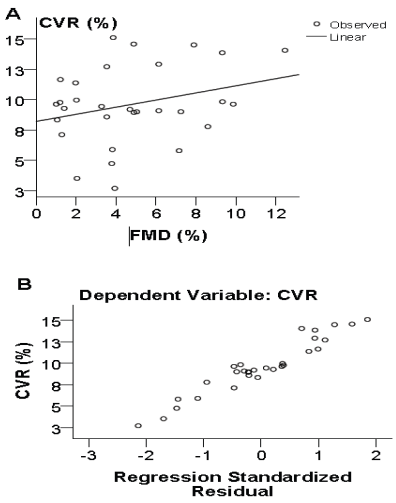

Fig. 3. Linear regression showing positive correlation between cerebrovascular reactivity to L-arginine (CVR) and flow-mediated dilatation (FMD) (A) and the display of regression standardized residuals (B) in patients with leukoaraiosis

Conclusions

Our pilot study revealed diminished CVR and FMD correlating positively in patients with LA compared to patients with similar VRF but without LA. This may reflect "intrinsic" generalized endothelial dysfunction regardless of VRF in patients with LA, which could be the initial, primary event in its pathophysiology. This may offer streamlined opportunities for future studying of clinical interventions aiming at enhancing endothelial function in patients with LA.

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The interest of post-mortem MRI for the detection of cortical micro-infarcts in neurodegenerative and vascular dementia syndromes

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Abstract:

7.0 –Tesla magnetic resonance imaging (MRI) is the only reliable technique to detect cortical micro-infarcts (CoMIs). As this MRI technique is not much available for in-vivo studies until now, we present a post-mortem 7.0-Tesla MRI study of CoMIs in brains with neurodegenerative dementia syndromes and with vascular dementia (VaD). CoMIs of different sizes were predominantly found in patients with cerebral amyloid angioparthy (CAA) and in VaD brains. In contrast cerebellar CoMIs were mainly due to atherosclerotic vascular disease.

*Keywords:*Post-mortem 7-Tesla MRI- Cortical micro-infarcts- Neurodegenerative diseases- Vascular dementia.

Introduction

On 1.5-tesla magnetic resonance imaging (MRI) cortical micro-infarcts (CoMIs) are considered as barely visible lesions in clinico-radiological correlation studies [1], while with 3.0-tesla MRI their detection level still remains low [2]. Even on naked eye examination of post-mortem brain sections these lesions are not easy to be found [3]. Recently, detection of CoMIs has become possible with high-resolution 7.0-tesla MRI in-vivo [4]. They have already been demonstrated in a limited number of elderly patients [5].

The present study investigates with 7.0-tesla the frequency of CoMIs in different post-mortem brains of patients suffering from neurodegenerative and vascular dementia syndromes.

Materials and methods

Hundred-seventy five post-mortem brains, composed of 37 with pure Alzheimer's disease (AD), 12 with AD associated to cerebral amyloid angiopathy (AD-CAA), 38 with frontotemporal lobar degeneration (FTLD), 12 with amyotrophic lateral sclerosis (ALS), 16 with Lewy body disease (LBD), 21 with progressive supranuclear palsy (PSP), 18 with vascular dementia (VaD) and 21 controls were examined.

Three coronal sections of a cerebral hemisphere from each brain were submitted to MRI: one of the frontal lobe at the level of the head of the caudate nucleus, a central one near the mamillary body and one at the level of the occipital lobe. In addition one horizontal section of a cerebellar hemisphere was examined to detect cerebellar cortical micro-infarcts (CeCoMIs).

We used a 7.0-tesla MRI Bruker BioSpin SA with an issuer-receiver cylinder coil of 72 mm inner diameter (Ettlingen, Germany). The samples were placed in plastic box, filled with salt-free water, after cleaning the formalin fixation. A positional, a spin-echo T2 and a gradient-echo T2* sequences were performed [6].

According to their size several types of CoMIs were detected on the 3 coronal sections of a cerebral hemisphere with 7.0-tesla MRI and compared to the mean CoMI load observed on histological examination of one standard separate coronal section of a cerebral hemisphere at the level of the mamillary body. Also the number of CeCoMIs was determined on a horizontal section of a cerebellar hemisphere for MRI examintion and on a separate section for neuropathological comparison.

Results

The MRI examination did not reveal any statistical difference of the mean values of CoMIs comparing adult, middle old and oldest age groups.
Overall CoMIs were significantly prevalent in the brains with neurodegenerative and cerebrovascular diseases associated to CAA compared to those without CAA. VaD, AD-CAA and LBD brains had significantly more CoMIs compared to the controls.
As the cortical arterial angioarchitecture is rather complex, the infarcts were of different size according to the occluded cortical perforating arterial branch. The CoMIs were composed of large ones, involving all cortical layers, intermediate ones affecting more than half of the cortical mantle and small ones only present in one or two cortical layers. While all types of CoMIs were increased in VaD and AD-CAA brains, a predominance of the smallest ones was observed in the LBD brains. In VaD the superficial cortical layers were by far the most frequently affected [7].
On the other hand CeCoMIs were approximately all of the same size, due their simple cerebellar cortical arterial angioarchiture, consisting of only one single type of perforating branches. CeCoMIs were only significantly increased in the VaD brains. When comparing the diseased patients with and without CAA mutually and to those with and atherosclerotic cerebrovascular disease and severe arterial hypertension only in the latter a statistically significant increase of CeCoMIs was observed [8].

Conclusions

Post-mortem 7.0-tesla MRI allows the detection of several types of CoMIs and their contribution to the cognitive decline in different neurodegenerative and cerebrovascular diseases. They are predoninantly observed in VaD brains. In contrast to the cortical micro-infarcts in the cerebral hemispheres that are mainly related to CAA, those in the cerebellum are more frequently due to atherosclerotic cerebrovascular disease.

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Post-mortem morphological imaging in neurodegenerative and vascular dementia syndromes

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Abstract

Magnetic resonance imaging (MRI) is a new technique that can be useful in the post-mortem examination of patients with neurodegenerative and vascular dementia syndromes. In particular, 7.0 T MRI allows to detect even lesions that are not visible on naked-eye examination.

Keywords:Post-mortem 7.0-Tesle MRI- Applications- Cerebral atrophy- cerebrovasular micro-lesions- Iron deposition.

Introduction

7.0-tesla magnetic resonance imaging (MRI) can be used as an additional tool to examine post-mortem brains of patients with neurodegenerative and vascular dementia syndromes [1]. It shows the degree and the distribution of the cerebral atrophy. It detects lesions that can be selected for histological examination. Small cerebrovascular lesions can be quantified and the iron load in the deep brain nuclei evaluated.

Methods and Materials

Formalin fixed brains may be used. In order to visualize the cerebral changes coronal sections of the cerebral hemisphere, sagital sections of the brain stem and horizontal sections of the cerebellum are the most appropriate. The sections are placed in a plastic box, filled with salt-free water after cleaning the formalin fixation. A MRI 7T Bruker Biospec (Ettlingen, Germany) can be used to perform a positional, a spin echo T2 and a T2*-weighted gradient-echo sequence [2]. Three up to 6 serial sections of a cerebral hemisphere and one section of brain stem and cerebellum allows to evaluate the most important brain changes and to select the small samples used for histological diagnostic purposes. Hundred-seventy five post-mortem brains, composed of 37 with pure Alzheimer's disease (AD), 12 with AD associated to cerebral amyloid angiopathy (AD-CAA), 38 with frontotemporal lobar degeneration (FTLD), 12 with amyotrophic lateral sclerosis (ALS), 16 with Lewy body disease (LBD), 21 with progressive supranuclear palsy (PSP), 18 with vascular dementia (VaD) and 21 controls were examined. A separate standard coronal section of a cerebral hemisphere was used to quantify the neuropathological lesions and validate the MRI findings [3].

Results

Comparison of 3.0-tesla to 7.0-tesla MRI, performed in a few cases with large haemorrhages, showed a larger extension of the lesions in the latter.

The degree of atrophy correlated well with the severity of histological lesions.

The detection of small bleeds on 7.0 T MRI was reliable for 96% in the cerebral cortex and less than 50% in the deep cerebral structures. Due to the blooming effect, not only micro-bleeds could be detected but also mini-bleeds, who were not visible on naked eye examination. However, the size of the small bleeds on MRI did not allow the differentiation between micro- and mini-bleeds, as the blooming effect differs from one specimen to another according to their composition and degree of haemosiderin deposition [4]. The small cortical bleeds predominated to a different degree in the frontal areas of all neurodegenerative disease groups and also of the controls. The highest incidence was found in CAA brains. In AD-CAA brains micro-bleeds were more frequent in the inferior parietal gyrus, the precuneus and the cuneus, compared to pure AD brains. In VaD brains they were more prominent in the prefrontal and postcentral regions [2]. Cortical microbleeds were mainly observed in

the deep cortical layers [5]. They were also observed in pure neurodegenerative diseases such as FTLD [6] and LBD [7].

Cortical micro-infarcts were by far more frequent in VaD and to a lesser degree in LBD and AD-CAA brains [3]. Cerebellar micro-infarcts on the other hand were mainly due to atherosclerotic disease rather than to CAA [8].

Lacunes and white matter changes were mainly observed in VaD brains. The latter were also frequently seen in AD-CAA and FTLD. However white matter changes in FTLD were due to Wallerian degeneration, rather than caused by cerebrovascular disease [9].

Superficial siderosis was due to haemosiderin deposits in the subpial space. It was not only caused by small cortical bleeds but also as frequently associated to small cortical infarcts as the latter had a more superficial location than the microbleeds [10].

Iron deposition in the claustrum, the caudate nucleus, the putamen, the globus pallidus, the thalamus and the subthalamic nucleus was significantly more observed in FTLD than in the other neurodegenerative diseases and in VaD. In AD brains only some increased iron content was present in the caudate nucleus. No differences were seen in the hippocampus, the mamillary body, the geniculate body, the substantia nigra, the red nucleus and the dentate nucleus between the different neurodegenerative and vascular dementia brains [11].

Conclusions

7.0-tesla MRI is indeed a useful additional tool in neuropathology of dementia syndromes. Not only the impact of small cerebrovasular lesions can be assessed but also other degenerative changes.

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The contribution of non-invasive brain stimulation techniques in the experimental treatment of cognitive and neuropsychiatric symptoms in Vascular Dementia

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Abstract

Repetitive Transcranial magnetic stimulation (rTMS) and transcranial Direct Current Stimulation (tDCS) are non-invasive and painless stimulation techniques able not only to explore cortical circuits and related neurochemical pathways in dementing illnesses but also to induce cortical plasticity with a potential therapeutic and rehabilitative purposes. Several studies, although methodologically heterogeneous and most of them open-label in design, have shown that specific paradigms of stimulation might improve cognitive performance, thus possibly becoming an alternative to conventional neuroleptic therapy for psychiatric symptoms of dementia. In Alzheimer's disease these effects are probably mediated by compensatory mechanisms supporting the residual abilities. The efficacy can be maximised by selecting patients on the basis of putative neurophysiological markers. Although less is known, similar plastic phenomena are invoked in Vascular Dementia (VaD). High frequency rTMS over the left dorsolateral prefrontal cortex improved executive performance in patients with subcortical ischemic vascular disease and the effect has been hypothesized to be due to an indirect activation of dopaminergic neurons in the mesencephalon and the noradrenergic and serototoninergic neurons in the brainstem. Moreover, rTMS was effective in alleviating also symptoms of vascular depression. More recently, the restorative effects of these techniques on cognitive ability have been observed in murine model of VaD probably through the neurotrophin release, such as the Brain Derived Neurotrophic Factor (BDNF) and the induction of hyppocampal glutamate-mediated synaptic plasticity.

Keywords: Cortical excitability, neuromodulation, synaptic plasticity, Vascular Cognitive Impairment, dementia.

Introduction

1.1 Background

Recently, repetitive Transcranial magnetic stimulation (TMS) and transcranial Direct Current Stimulation (tDCS), are emerging as promising tools to explore cortical circuits and probe different neurochemical pathways in dementing illnesses [1] [2] [3]. Although the abnormalities revealed by these non-invasive brain stimulation techniques are not disease-specific, some neurophysiological measures change consistently with the involvement of different neurobiological substrates in the pathogenesis of each disease, even in the earliest stages [4] [5]. Moreover, the ability to induce cortical plasticity in the motor cortex and connected areas underlies the interest in therapeutic and rehabilitative application in several neuropsychiatric disorders, such as major depression (MD) [6].

1.2 Physical principles and technical considerations

As known, conventional TMS for research purpose is performed using a figure-of-eight shaped coil placed on the subject's head through a mechanical coil holder. A brief electrical current (μs) generates a magnetic field

around the coil windings, which, in turn, induces electrical currents in the brain that flows in parallel but opposite to those in the TMS coil. In the case of tDCS, two sponge electrodes (i.e., anode or cathode, depending on DC polarity) are fixed to the subject's head and a constant electrical current generated by the tDCS stimulator is usually applied over a few minutes (e.g., 3-20 min). The electrical current flows from the anode (+) to the cathode (-) through the superficial cortical areas, thus leading to polarization [4].

Based on these physical principles and technical considerations, these approaches can transiently influence the function of stimulated and connected areas, mainly depending on specific stimulation parameters. High frequency (> 1 Hz) rTMS and anodal tDCS transiently enhances cortical excitability, whereas slow frequency (≤1 Hz) rTMS or cathodal tDCS transiently depresses it. Therefore, since the effects of repeated sessions may persist in time, both rTMS and tDCS have a wide range of theoretical therapeutic and rehabilitative applications in different neurological and psychiatric disorders. The mechanisms underlying these changes are not clear, but seem to be related to synaptic long-term potentiation (LTP) and long-term depression (LTD) within the central nervous system [4]. For these reasons, in October 2008 the Food and Drug Administration (FDA) approved rTMS for the add-on treatment of drug-resistant major depression and gave the first instructions on how to use high-frequency rTMS. Transcranial DCS has been advanced to Class I level in patients with post-stroke motor rehabilitation, although, as known, Class I categorization is a measure of the strength of the results and does not necessarily translate into Level A recommendation for clinical practice.

Both techniques have pros and cons: TMS has the advantage to directly elicit action potentials and stimulate areas which are distant yet functionally connected; moreover, the manipulation of parameters can result in varied cortical effects and greater focality of stimulation. However, TMS is relatively expensive and it needs a degree of technical expertise; furthermore the magnetic coil is required to be held still, and sham (simulated) stimulation is challenging. Conversely, tDCS is less expensive, easily portable and safe, feasible to train within a home or clinical environment, can be better administered in synchrony with a cognitive task, and provide a reliable sham condition; nevertheless, tDCS has the disadvantage to do not directly elicit action potentials because it is dependent on the pre-existing neural state, and the effect is usually weaker than TMS (it only increases spontaneous cell firing) [3].

Neuromodulation in Dementia

2.1 Rationale and experimental hypothesis

Several studies, although methodologically heterogeneous, have shown that specific paradigms of stimulation might improve cognitive performance and became an alternative to conventional neuroleptic therapy for psychiatric symptoms of dementia. Current pharmacological treatment, indeed, have significant limitations, such as non-specific effects, insufficient tailoring to the individual, and moderate-severe adverse effects.

In this context, TMS has become increasingly popular, with its relatively ease of administration, non-invasivity, very few side effects and wide range of potential applications. It was initially used to non-invasively evaluate the integrity of the cortical-spinal tract in humans. Since these early studies, the development of paired-pulse and rTMS protocols allowed investigators to explore inhibitory and excitatory interactions of various motor and non-motor cortical regions within and across cerebral hemispheres. [1] [2]. These applications have provided insight into the intracortical physiological processes underlying the functional role of different brain regions in various cognitive processes, motor control in health and disease and neuroplastic changes during recovery of function after brain lesions [5]. Used in combination with other neurophysiological techniques and imaging tools, TMS provides valuable information on functional connectivity between different brain regions, and on the relationship between physiological processes and the anatomical configuration of specific brain areas and connected pathways [4].

Recently, rTMS and tDCS have been tested in dementing illnesses as innovative non-pharmacological strategies for the treatment of cognitive and mood-behavioural symptoms [3]. The targets for an ideal treatment would be: a) modulation of activity in the targeted cortex; b) modulation of activity in a dysfunctional network; c) restoration of adaptive balance in a disrupted network; d) guiding plasticity for best behavioural outcome; e) suppression of maladaptive changes for functional advantage.

2.1 Alzheimer's disease

In Alzheimer's disease (AD), there is a general trend for improvements across a wide range of cognitive outcome measures following treatment with rTMS and tDCS, probably mediated by compensatory mechanisms supporting the residual abilities, even with long-lasting effects [3]. Typical sites of stimulation include the dorsolateral prefrontal cortex (DLPFC), temporal regions, temporo-parietal regions or a combination of multiple regions. Interestingly, the benefits may be highly task-specific (i.e. action naming *vs.* object naming, visual recognition *vs.* spatial recognition) and sustained, taking into account that dementia severity may affect the clinical response. Combining these techniques with cognitive rehabilitation might influence learning in a neuroplastic fashion.

2.2 Vascular Dementia

Although less is known, plastic phenomena are also invoked to take place in patients with Vascular Dementia (VaD) [7]. Motor cortex has been found to have an enhanced excitability in patients with both AD and subcortical ischemic VaD with respect to controls, and it was plastically rearranged [1] [2]. Moreover, a significant direct correlation between parameters associated to cortical excitability and those associated to cortical plasticity was evident, suggesting the existence of mechanisms that partially overlap and probably act in the same neurophysiological way although they are, at least in principle, different both in localization (subcortical *vs.* cortical) and in origin (vascular *vs.* degenerative) [7]. Therefore, AD and subcortical ischemic VaD might share a common neurophysiological platform, related to the progressive neuronal loss within motor areas and to the ischemic disconnection, respectively. This alteration finally could promote the observed functional rearrangement that could allow the preservation of motor programming and execution despite disease progression [7].

In a randomized controlled pilot study on patients with subcortical ischemic vascular disease and clinical picture of Vascular Cognitive Impairment-No Dementia (VCI-ND), Rektorova and colleagues [8] showed that high frequency rTMS over the left DLPFC improved executive performance and hypothesized that long-lasting effects could be due to an indirect activation of monoaminergic neurons located in the mesencephalon (dopamine) and/or the brainstem (noradrenaline and serototonine) and their cortical and subcortical targets [8]. In the same class of patients, rTMS was able to alleviate depressive symptoms, suggesting a potential application even in individuals with vascular depression (VD) [9]. In depressed patients, white-matter hyperintensities and global vascular risk are predictors of poor response. Although the overall quality of the studies does not allow findings generalization, investigations of higher validity support the VD concept for interventions.

The efficacy of treatment can also be maximised by selecting patients on the basis of putative neurophysiological markers. For instance, TMS performed in VCI-ND patients revealed a pattern of cortical excitability characterized by an hyperfaciliation and a clear trend toward a progressive increase of global cortical excitability after 2-years follow-up [10] [11]. A similar electrocortical profile was observed in patients with VD [12] but not with MD [13]. Moreover, it was demonstrated that VD correlates with the leukoaraiosis and not with the lacunar infarcts and that the presence of executive dysfunction was predictive of poor outcome after pharmacological therapy [14]. This highlights the role of specific TMS parameters as candidate markers of disease process [10] and progress [11] and potential targets of the modulatory properties of brain stimulation techniques [15], supporting the concept of dementia as a dynamic condition. The enhanced cortical plasticity might counteract cognitive decline and shed light on the reasons underlying decline or preservation of cognitive domains [15].

2.3 Parkinson's disease

Regarding Parkinson's disease (PD) with cognitive impairment, as in the other dementias, methodological heterogeneity was apparent; however, the overall results suggest that rTMS might benefit depressive symptoms in patients with PD and that the positive effects may persist in time in some cases. Lack of studies using tDCS for cognitive symptoms in PD with or wihout dementia should be encouraged in order to examine any non-motor benefits [3].

2.4 Findings from preclinical models

More recently, intriguing findings come from preclinical studies, showing the restorative effect of rTMS on cognitive ability in murine model of VaD and its impacts on hyppocampal synaptic plasticity [16] [17]. Another possible mechanism of action of non-invasive brain stimulation in dementia is represented by the modulation of neurotrophin release. Indeed, experimental studies in rat models of AD have shown that tDCS can improve learning in mice through BDNF secretion and activation of tyrosine kinase B receptor [18] while low-frequency rTMS might improve cognitive deficits through the up-regulation of the hippocampal Brain Derived Neurotrophic Factor (BDNF) and the expression of the glutamate receptor for N-methyl-D-aspartate (NMDA) [19]. Finally, low-frequency rTMS in VaD model rats may improve learning and memory, protect pyramidal cells from apoptosis and promote hippocampal synaptic plasticity trough increased expression of the Bcl-2 and reduced expression of Bax [20].

2.5 Limitations

Among the studies here reviewed, overall it is evident that there is a great deal of methodological heterogeneity in the use of non-invasive brain stimulation. In order to advance the use of both techniques, the replication of studies is necessary give that: a) the majority of reported investigations are open-label or uncontrolled in their design; b) dementia patients are frequently on a range of psychotropic medications that can interact with the effects of TMS/tDCS; c) the treatment response could be affected by changes in brain morphology (i.e. cortical atrophy, CSF distribution); d) determining the most appropriate location for TMS or rtDCS is often challenging; e) deterimingn to what extent cortical response characteristics of the motor system are representative of other brain systems is not entirely known; f) there is a wide range of TMS and tDCS stimulation parameters which could be applied in dementia populations that need to be considered. Possible

solutsions consisted in: a) fully report of all trial results, including negative findings; b) more trials in healthy individuals, or in those with mild disease, allowing finessing of stimulation parameters and establishing the tolerability of protocols; c) further studies of the aetiological models of dementia, including preclinical ones, thus aiding the choice of stimulation site; d) optimization of the treatment efficacy through methods of stratification, where patients are selected on the basis, for instance, of neuropsychological, neurophysiological or genetics putative markers; e) use of novel methodological factors that may increase the stimulation efficacy (i.e. neuronavigated system, combination with hdEEG or fMRI, high-definition tDCS, etc…).

Conclusion

In conclusion, there is a mounting interest towards non-pharmacological therapeutic tools for cognitive rehabilitation in dementia, including VaD. Current data, although obtained from heterogeneous studies, have revealed that rTMS and tDCS can induce beneficial effects on specific cognitive domains and neuropsychiatric manifestations. Recent findings from animal models are exciting, but their clinical significance needs to be validated. Challenges exist in terms of appropriate patient selection and optimisation of the stimulation protocols. Together with the clinical assessment and the widely used diagnostic tools like neuroimaging, systematic neurophysiological assessment of dementing patients can aid the diagnostic process and predict the response to drugs.

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Risk factors of unfavorable course of cerebral small vessel disease – preliminary data from SHEF-CSVD Study

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Abstract

Natural clinical course of cerebral small vessel disease (CSVD) has not yet been thoroughly described. The aim of the single center, prospective study being a part of SHEF-CSVD Study was to establish risk factors of unfavorable course of CSVD in 24 months follow-up. 137 functionally independent patients with marked MRI features of CSVD and with recent lacunar stroke (n=47, LS), 28 with vascular Parkinsonism (VaP), 42 with vascular dementia (VaD), 20 with deep hemorrhagic stroke and 44 controls (CG) matched for age, sex and vascular risk factors were prospectively recruited. Brain MRI was performed at the study entry and after 24 month of follow-up. Unfavorable course was defined as occurrence of death, any vascular events or progression of MRI white matter lesions (WMLs) during observation. Blood markers of endothelial dysfunction and inflammation and cerebral vasomotor reactivity (VMRr) in response to hypercapnia using TCD examination were determined. Unfavorable outcome was more frequent in CSVD group vs CG (32% vs 9%, $p<0.01$). Independent of age risk factors associated with poor outcome were: baseline MMSE <23 p, VMRr <53%, hsCRP >0,69 mg/L, serum albumin level <3,45 g/dL, tissue factor >61, 75 ng/ml, severe WMLs (modified Fazekas score ≥2p), lack of statin and aspirin use, no physical activity and nocturnal blood pressure fall.

Keywords: small vessel disease, prognosis, hemodynamic and hemostatic risk factors

Introduction

Cerebral small vessel disease (CSVD) is a syndrome of clinical, cognitive, neuroimaging and pathological findings arising from brain damage in the cerebral white and deep gray matter [1]. Cerebral small vessel disease leads to recurrent lacunar strokes (LS), deep haemorrhagic strokes (HS) and also gait disturbances, vascular dementia (VaD) and vascular parkinsonism (VaP) but long-term course and prognosis of the disorder are not well known [2]. Although associated with vascular risk factors like increasing age, hypertension and considered to result from cerebral arteriolar occlusive disease, the pathogenesis is largely unknown [3]. It probably starts with an increase in permeability of the blood-brain barrier caused by endothelial dysfunction. Endothelial cell dysfunction can be assessed in vivo by measuring circulated molecules of endothelial origin or molecules interacting with endothelial cells but also by assessing cerebral vasomotor reactivity reserve (VMRr). We hypothesized that these markers of endothelial dysfunction can be more reliable than vascular risk factors in predicting course of different manifestations of CSVD.

Aim

The aim of the single center, prospective study was to establish risk factors of unfavorable course of CSVD in 24-month follow-up as a part of SHEF-CSVD Study (Significance of HEmodynamic and hemostatic Factors in the course of different manifestations of Cerebral Small Vessel Disease) [4].

Materials and Methods

The study group consisted of 137 patients aged between 60 and 90 years with radiologically confirmed CSVD: with recent LS (n=47), HS (n=20), established VaP (n=28), VaD (n=42), and 44 controls (CG) matched for

age and vascular risk factors. The patients were prospectively recruited between December 2011 and May 2013 and hospitalized in Clinic of Neurology, Military Institute of Medicine, Warsaw, Poland. The study protocol and methods have been thoroughly described elsewhere [4]. In brief, CSVD group consisted of physically independent patients (mRS<3, NIHSS<10 points) without severe dementia (MMSE≥12 points) with extensive (≥2 points in Fazekas scale) CSVD on 1.5T MRI neuroimaging with typical white-matter lesions (WMLs) or periventricular hyperintensities (PVH) visualized on T2 and PD/FLAIR images. CSVD groups were diagnosed according to typical radiological and clinical picture: LS - according to the OCSP Criteria, HS –hemorrhage to deep structures of the brain: the thalamus, basal ganglia, pons after exclusion of vascular malformations or coagulopathies; chronic VaP - according to Hurtig scale; VaD – basing on Modified Hachinski Ischemic Scale ≥ 7 points and fulfilling NINDS-AIREN criteria [5,6,7]. To prevent confounding by hyperacute phase responses, all LS and HS patients underwent study procedures at least 2 weeks after their index strokes (mean 16±2 days). VMRr measurements were performed with the use of a transcranial Doppler techniques: mean flow velocities (MFV) were measured in patients in a supine position in a quiet and breathing room air via transtemporal insonation of both MCA with a 2-MHz probes in a fixed position and averaged. TCD measures were performed at rest with normal breathing (normocapnia), after hyperventilation (hypocapnia) and hypoventilation (hypocapnia) according to the Markus and Harrison procedure, in which patients are instructed to hold their breath for 30s in one inspiration after a period of 4 minutes of normal breathing of room air. VMRr was calculated as the percentage change in the MFV according to $VMRr = (MFV_{hypercapnia} – MFV_{normocapnia})*100/MFV_{normocapnia}$) [8,9]. To minimize the effect of confounding factors VMRr was assessed when subjects were fasted and had avoided exercise, caffeine and medication intake for at least 6 h. Blood was sampled from the antecubital vein into 5-ml serum plasma tubes and was measured with commercially available ELISA kits according to the manufacturer's instructions (Bio Source, Europe, Nivelles, Belgium). All of the clinical assessment (neurological, radiological, ultrasound) were performed by the 2 "blinded" assessors, unaware of the clinical diagnosis. All participants from both groups signed an informed consent form. This study was approved by the local Medical Ethics Committee and was supported by the Polish Ministry of Science and Higher Education as a research project of the Military Institute of Medicine (Warsaw, Poland, study number N N402 473840). Log normal data were compared using paired t tests, non-normal data were analyzed using non parametric tests, the chi-square test was used for comparisons of categorical variables. The analyzes of correlations were performed by bivariate correlation analysis. A probability value of $p<0.05$ was considered significant. All data are presented as mean±SD values. All analyses were performed using Statistica 12 software (StatSoft Inc, USA).

Results

Mean age (73±6 vs 71±5 years, p=0,2), sex distribution (males: 52% vs 60%, p=0,4) and vascular risk factor profiles and other patient characteristics, frequency of use of antihypertensive agents were similar among CSVD and CG although patients with LS and VaD more often received statin and antiplatelets compared to the controls (Table 1).

Table 1. Characteristics of lacunar stroke patients, vascular parkinsonism and vascular dementia patients, and control subjects.

	Lacunar stroke patients (n=47)	**Deep hemorrhagic stroke** (n=20)	**Vascular parkinsonism patients** (n=28)	**Vascular dementia patients** (n=45)	**Control subjects** (n=44)
Age, mean years (±SD)	70±7	74±9	72±6	74±6	71±5
Male sex %	48	50	64	35	60
Hypertension	90	98	71	84	70
Coronary artery disease	18	15	11	25	11
Diabetes mellitus	51	45	50	25	40
Current smoking	60	60	54	51	50
Hypercholesterolemia	77	55	71	21	65
Statin use	80*	50	58	70*	51
Antiplatelet use	95*	20	56	71*	44
Antihypertensive use	90	94	81	91	70

Data are % except where otherwise noted
*Significant difference between studied group vs control subject ($p<0.05$).

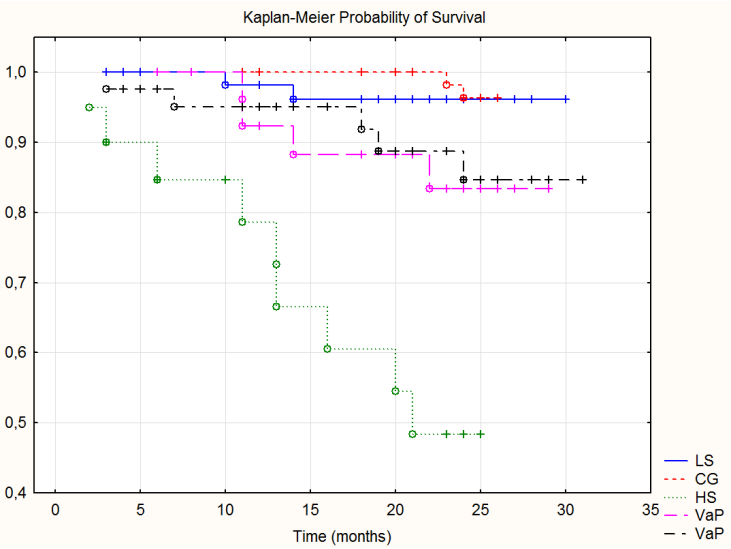
The overload of WMLs was similar among CSVD groups (mean Fazekas score of 2). Patients with CSVD compared to CG had significantly decreased VMRr (51±0,05 vs 71±0.17%, p<0,001) and serum albumin level (3,7±0,6 vs 4,4±0,5 mg/dL, p<0,001), increased hsCRP (0,9±1,02 vs 0,13±0,12 mg/L; p=0,01), and tissue factor (TF) activity (52,7±18,1 vs 44,3±18,1 ng/mL, p=0,04). There was also a trend towards increase level of uric acid in CSVD compared with CG (5,9±1,7 vs 5,4±1,1, p=0,08). Detailed data in separate CSVD groups are presented in Table 2.

Table 2. Mean level of VMRr, hsCRP, albumin,tissue factor and WMLs load classified by study groups.

	Lacunar stroke patients (n=47)	**Deep hemorrhagic stroke** (n=20)	**Vascular parkinsonism patients** (n=28)	**Vascular dementia patients** (n=45)	**Control subjects** (n=44)
VMRr (%)	0,51±0,18*	0,44±0,14*	0,58±0,17*	0,51±0,1*	0,71±0,17
hsCRP (mg/L)	0,80±2,26*	2,43±0,14*	0,74±0,9	0,63±1,1*	0,15±0,15
albumin (mg/dL)	3,71±0,74*	3,65±0,47*	3,97±0,6*	3,60±0,89*	4,42±0,51
Fazekas score	2,11±0,66*	2,3±0,5*	2,10±0,7*	2,10±0,56*	0,5±0,5
Tissue factor (ng/mL)	49,3±11,96	44,01±19,44	46,70±16	109,11±191	44,34±18,15

*Data are means with SD. Statistically significant differences between CSVD and CG groups are marked * (p<0.05).*
Key: VMRr - cerebral vasomotor reactivity reserve; hsCRP – high sensitivity C-reactive protein

Basing on Kruskal-Wallis ANOVA and median test no statistically significant difference in endothelial serum markers and VMRr between CSVD groups were noted (p>0,05). Unfavorable outcome was more frequent in CSVD group vs CG (32% vs 9%, p<0.01; Picture 1). Independent of age factors associated with poor outcome were: baseline MMSE (odds ratio, OR 0,84; p<0,001), VMRr (OR 0,07; p<0,01), hsCRP (OR 1,08; p<0,01), albumin level (OR 0,56; p<0,05), TF (OR 1,01; p=0,04), severe WMLs (OR 1,6; p=0,04), statin (OR 0,4; p=0,04) and aspirin use (0,29; p=0,02), physical activity (OR 0,45; p<0,001) and lack of nocturnal blood pressure fall (OR 3,8, p=0,01). The calculated cut off points were: MMSE <23 p, VMRr <53%, hsCRP >0,69 mg/L, serum albumin level <3,45 g/dL and TF >61, 75 ng/ml.



Picture 1. Kaplan-Meier probability of survival free of vascular events and death in study groups.

Discussion

This is the first study that has simultaneously evaluated VMRr and serum markers reflecting endothelial function in a well-phenotyped cohort of CSVD patients in comparison to control group. It has been unknown so far if these markers simply reflect exposure to vascular risk factors [10]. The main outcome of our study is that, in comparison with controls matched for age, sex, and vascular risk factors patients with clinically significant CSVD had higher levels of serum markers and weaker vascular reactivity indicating more pronounced

endothelial dysfunction. Furthermore, there was no significant difference in analyzed factors between patients with different clinical manifestation of severe CSVD indicating similar level of endothelial dysfunction in these states. The study demonstrated that hemodynamic and hemostastic data can be used as early predictors of the unfavorable radiological and clinical course of different clinical manifestations in CSVD. Our findings suggest that low-grade inflammation (as assessed by hsCRP) and endothelial activation (measured by tissue factor) and probably brain blood barrier disintegration (indicated by low albumin level as an effect of leakage of albumin) are associated with cerebral microstructural disintegration or other pathologic changes in CSVD regardless of the different clinical manifestation. Low level of serum albumin may also be a marker of chronic systemic inflammation, whereas in physiological concentrations, serum albumin may act as an anti-inflammation protein and antioxidant. The study also demonstrated a decreased response in VMRr in CSVD what provided data on the impaired capacity of the endothelium to increase the bio-availability of NO and decreased microcirculation flow at least associated with critical hypoperfusion. We demonstrated similar level of endothelial dysfunction in diverse manifestations of CSVD with comparable WMLs burden. The underlying mechanism is thus pathophysiologically extensive, chronic and involves the whole vascular bed. The pathomechanism responsible for different clinical picture of CSVD however remains unknown. Our study has some limitations. The major weakness is the potential for random error or selection bias because of the small number of patients and controls included. The strengths of our study remain that we simultaneously studied a group of well characterized patients with different clinical picture of CSVD and control population sharing similar risk factor and treatment profiles.

In conclusion, we have shown that similar impairment of the cerebral microvasculature and endothelial function is present in different manifestations of CSVD compared to vascular risk factors matched control group. Screening of cerebrovascular reserve by measuring VMRr using TCD could represent a much-needed, reliable, safe, and cost-effective technique for patients at with CSVD. Future studies should check the validity of these experimental and hypothesis-generating pilot results.

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Large Mammals - translational stroke models in sheep

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Abstract

International academic and industrial expert consortia (STAIR, STEPS) suggest stringent preclinical research strategies for stroke including validation of novel treatment strategies in gyrencephalic animal models. However, many existing gyrencephalic animal models are expensive, constrained by ethical considerations or limited by species-specific anatomical restrictions. Importantly, many large animal models do not allow long term investigations, being crucial for robust safety and efficacy assessments.

To overcome these limitations, an ovine model of cerebral ischemia by permanent middle cerebral artery occlusion (MCAO) in sheep was established. After detailed characterization of the model itself, it was utilized in acute stroke treatment studies, for example using nitrogen monoxide, but also in long term experiments such as assessment of autologous bone marrow cell therapy. Additionally, frameless stereotaxic methods were established that enable local administration of cells and/or substances for different purposes. Moreover, the ovine model is routinely used for state-of-the-art imaging studies (MRI, PET and combined PET/MRI) including automatic image processing and analysis using ovine stereotaxic atlas.

Major strengths of the ovine MCAO model comprise reproducible lesion size, capability for long term studies and suitability for clinical imaging procedures, as well as relatively low costs. The transcranial approach avoids enucleation, enabling testing of neurological functions without modelling effects, but at the cost of artificial intracranial pressure profiles. The model has also been adopted for transient MCAO.

Keywords: sheep, MCAO, ischemia, translational studies, Nitric Oxide, Bone Marrow, brain atlas

Introduction

There is a need for refinement of research strategies in the field of cerebrovascular diseases including vascular cognitive impairment diseases (VCID), which is currently dominated by rodent models. This includes translational aspects which may not only (1) require new models imitating human VCID in a different biological environment, but also (2) need to consider physiological and anatomical factors as well as co-morbidities closer to the human situation. Compared to rodents an obvious benefit of large species is the option to apply more complex imaging-based investigations *(in-vivo)* under clinically relevant conditions and using clinical equipment. Here, the size and complexity of large animal brains allows for detailed investigation of particular structures and brain areas, but also functional imaging of cerebral metabolism. Therefore, the inclusion of large animal models in a particular research program comes with a number of advantages which have been exemplified in related fields. For example, such models are used to investigate acute and chronic cerebrovascular disorders including stroke. Here, we present a large animal model of Middle Cerebral Artery Occlusion (MCAO) [1] in sheep which includes physiological and anatomical comparability [2], and enables various relevant techniques and approaches for cerebrovascular research in gyrencephalic brain.

Model specification of the ovine MCAO

Effective stroke treatment by thrombolysis or recanalization is limited to relatively narrow time windows, still excluding most patients. Alternative pharmacological approaches, successfully tested in rodents, have subsequently failed in clinical trials. The most likely reason may be a translational gap in preclinical research design, particularly missing the inclusion of species with gyrencephalic brains [3]. Existent large animal models (dog, primate, cat, pig) exist, but exhibit considerable limitations such as ethical drawbacks (especially primates), high experimental and maintenance costs, as well as limited post-stroke observation times. Therefore, we introduced a simple, but reliable stroke model using MCAO in sheep ([1]).

The method is described in detail by [1]. Briefly, after a pretest period about 14 days including vaccination, treatment with antiparasitics, as well as clinical and neurological baseline investigations, healthy adult hornless Merino rams were randomly assigned to sham (no-occlusion but craniotomy), 1-branch, 2-branch and total MCA occlusion. Transcranial surgery and selective occlusion of the left MCA according to the group assignment were performed at day 0 in general anaesthesia. All animals were allowed to wake up afterwards and were integrated in the flock after 2 day of isolated convalescence. Imaging procedure using positron emission tomography (Tracer: ^{15}O-H_2O, ECAT EXACT HR+; Siemens/CTI, US) and magnet resonance tomography (MRI, 1.5 T Philips, Netherlands) were performed at day 1, 14, 42 [4]. Neurological deficits were determined on day 0 to day 42 and include hopping and hemistanding reaction, torticollis and circular movement. All animals were sacrificed at day 49 followed by neuropathological examination.

Diffusion (via diffusion weighted MR imaging) and perfusion (via ^{15}O-H_2O PET, see Fig. 1A) deficits were most prominent after total MCAO, decreased in 2-branch MCAO and minimal in 1-branch MCAO animals but could not be detected in in sham animals. Hemispherical atrophy (HA, ratio ipsi-/contralateral tissue volume) was not significantly different between the MCAO types at day 14. At day 42, it was largest in total MCAO, less pronounced in 2-branch and minimal in 1-branch MCAO animals ($p<0.05$). Animals with total MCAO showed most prominent functional deficits during the complete study while significant differences between 1-branch and 2-branch MCAO group were detected only until day 10 but not thereafter. Sham-operated animals completely recovered within 4 days. After sacrifice, a local, chronic territorial infarct was detected in all MCAO animals apart from sham-operated subjects. A massive infiltration with macrophages, vessel-associated type-1 and reticular type-3 collagen was evident in the infarcted area while next to the infarction glia scar formation occurred. A mild astrogliosis and activated microglia could be also observed in more remote brain tissue.

Although a *rete mirabile epidurale rostrale* necessitates a transcranial approach to access the MCA in sheep, the model enables robustly studying stroke after hemicraniectomy. The ovine MCAO model enables the induction of reproducible lesion sizes and neurological deficits. Moreover, the long term study ability and applicability of clinical scanners such as combined PET/MRI foster multi-parametric longitudinal study designs.

Enhanced cerebrovascular perfusion in acute stroke scenario

Rescuing the ischemic penumbra, the viable tissue surrounding the nonviable infarct core, remains a central aspect for treatment strategies in acute stroke. In this area, blood flow is critically reduced but still suffices to sustain neuronal integrity for hours. This offers a therapeutic time window for several treatment strategies. [5] showed that iNO preferentially dilates arterioles in areas of low perfusion. Therefore, iNO improves metabolic function and prevents the hypoxic penumbra from necrosis. The ovine MCAO model was chosen to validate these findings in a clinically relevant scenario.

The method is described in detail by [5]. Briefly, adult female Merino sheep were subjected to total MCAO. Baseline PET imaging was performed in persistent general anaesthesia afterwards. Two hours after induction of MCAO, randomly chosen animals received 50 ppm of iNO for 60 minutes, whereas controls were ventilated normally. Sheep were subjected to follow-up serial ^{15}O-H_2O PET scans 30min, 55min after start of iNO (control: air) as well as 30min after termination of iNO/air application. The last measurement was followed by MRI. The penumbra was identified by absolute CBF measurements (thresholds: 8–22 mL/100 g× min).

iNO selectively increased CBF in the ischemic penumbra as compared to both, the baseline condition before iNO application and control treatment (air). Importantly, quantification of penumbral CBF before and after iNO showed that about 50% of the penumbral volume turns into normally perfused tissue during iNO application, but remains unchanged in controls ($p<0.05$ vs baseline and vs control)(see Fig 1B). An oxygen saturation drop or partial carbon dioxide increase during this study could not be detected. A mild increase of methemoglobine could be observed only during iNO application, but was swiftly reversed after termination.

The study suggests that iNO exerts the properties of a promising therapeutic agent selectively targeting the penumbra without hemodynamic effects on normally perfused tissue. Since NO is already approved for human use [6], its clinical evaluation for ischemic conditions in which collateral blood flow is important, e.g. in stroke, is a feasible approach.

Autologous transplantation of Bone Marrow derived Mono-Nuclear Cells (BM MNC)

The efficacy of cell transplantation was demonstrated in rodent stroke models using various sources of adult stem cells. Bone marrow (BM) is an easy approachable source of different cell types including stem cells, and is capable to induce regeneration following stroke even beyond therapeutic time windows for recanalization. The mononuclear cell (MNC) fraction is most often applied experimentally. However, this treatment strategy has not been tested yet in the gyrencephalic brain. Therefore, an autologous transplantation approach in the ovine MCAO model was chosen to verify efficacy and practicability in a clinically relevant scenario.

Hornless Merino rams were randomly assigned for control (no treatment, N=15) and BM MNC transplantation (N=16). After pretest period about 14 days, 31 animals were subjected for total MCAO (day 0). Neurological baseline was determined (day 1) followed by BM aspiration from iliac crest, baseline MRI and PET and, finally, intravenous administration of purified BM MNC (>4.0x10^6 autologous BM MNC per kilogram body weight). MR and PET imaging was performed at days 1, 14 and 42. Neurological deficits were investigated from day 1 to 42. All animals were sacrificed at day 49 followed by pathohistological analysis.

There were no statistical significant differences in neurological deficits between the groups until d7 following MCAO ($p>0.05$). Thereafter, BM MNC treated animals showed an improved functional outcome compared to the control group ($p<0.05$). HA was significantly reduced in BM MNC group ($p<0.05$) while CBF defect volume enlarged significantly in control, but not in BM MNC treated animals ($p<0.01$). A decreased tissue loss in the BM MNC group could be confirmed post mortem ($p<0.01$). Immunohistological staining with GFAP revealed an increased astrogliosis next to the infarction in BM MNC animals which was reduced in the remote tissue, both compared to control animals ($p<0.05$). Further, lymphocytic infiltration and axonal degeneration were reduced in BM MNC group compared to control (see [7] for more details).

Considering results of this translational study and findings described in literature, autologous BM MNC transplantation may be a viable treatment option beside the therapeutic time window. Clinical studies are ongoing to test the efficacy and practicality in patients suffering from ischemic stroke [8] according to [9].

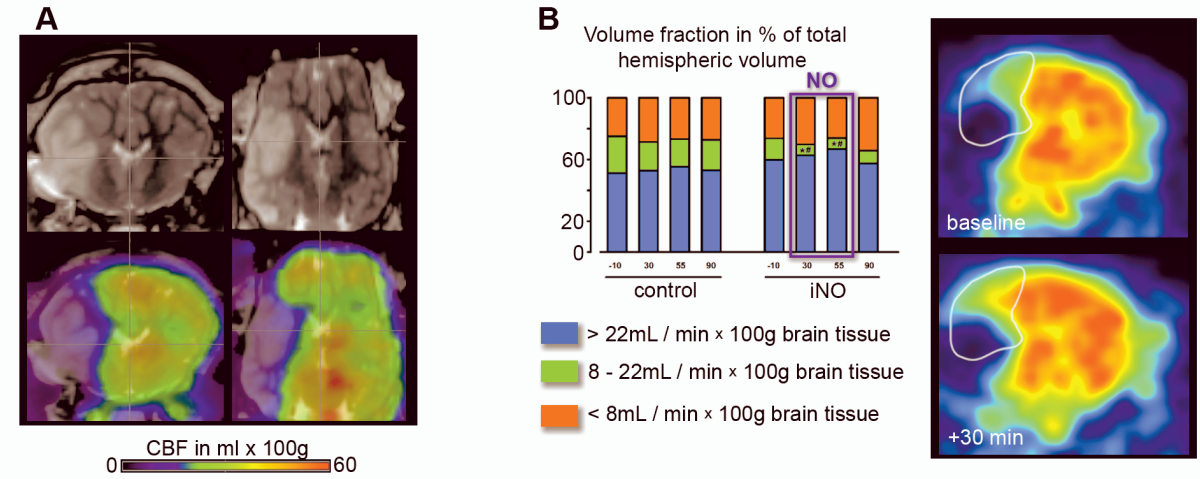

Fig. 1: *Imaging stroke in sheep.* ***(A)*** *The territorial infarction appears hyperintense in t2w TSE MRI (top) after 14 days while cerebral blood flow (CBF) via* ^{15}O-H_2O *PET-Tracer were strongly decreased in the ischemic area (bottom).* ***(B)*** *The penumbra volume (green) is significantly decreased in favour of normally perfused tissue (blue) in iNO group. The core infarct volume (red) remained stable until the end of application (bottom). Visualisation of PET* ^{15}O-H_2O *CBF revealed an increased perfusion in cortical areas 30min after initiation of NO-application. Original images can be found in [5]*

Image processing in an autologous hemorrhagic stroke model

Advantages of gyrencephalic large animal models in the field of translational neuroscience include the capability for iterative imaging procedures using clinically relevant techniques. Species-specific stereotactic standards such as MR brain atlases are necessary for automatic image processing, to enable a strongly recommended objective and observer-independent image analysis. However, statistical image analysis requires an adaptation of established frameworks for each species including templates and segmentation procedures. Here, structural MR data sets of a recently developed intracerebral haemorrhage (ICH) model in sheep were chosen to establish an analysis pipeline for the lesioned sheep brain by using the standard ovine brain template in Statistical Parametric Mapping (SPM).

The data include MR sequences (t1 3D, t2w TSE, FLAIR) before and after autologous, stereotactically induced ICH in sheep [10]. Image preprocessing was performed using FisImageJ (FIJI) for manual processing and Statistical Parametric Mapping 8 (SPM8 with Matlab R2010) including the VBM8 toolbox for automatic image processing. Tissue classes for grey (GM) and white matter (WM) as well as cerebrospinal fluid (CSF)

which are aligned with the standard stereotaxic sheep brain template [11] were modified by subtraction of the manually delineated lesion probability. The new generated tissue probability maps including GM, WM, CSF and lesion were implemented in SPM8 followed by volume calculation of the segmented tissue masks. Further, voxel-based morphometry (VBM) was performed to analyse the effect of the bleeding on lateral ventricle.

The automatic segmentation with SPM8 reproduced high-contrast tissue masks without remarkable misclassified voxels. As expected, volumes of GM and WM were decreased after cerebral bleeding. Interestingly, the ratio between ventricle to brain tissue was significantly higher after bleeding in the contralateral hemisphere while VBM revealed a significant compression of the lateral aspect of the ipsilateral lateral ventricle in comparison to initial ventricle volume but an expansion of the olfactory bulb ventricle ($p<0.01$).

Standard automatic image processing and analysis of ovine image data using SPM is feasible. The pipeline avoids laborious and subjective manual image processing procedures. Further, the advantages of voxel-based-morphometry can be used to determine group-specific or longitudinal differences. Further work is required to adapt the procedure for MCAO model in sheep.

Discussion

In summary, large gyrencephalic animals including sheep provide relevant advantages fostering translational study designs. It is obvious that methods are required to adapt the species into a VCID research such as experimentally induced hypertension [12]. Although drawbacks (e.g. *rete mirabile epidurale rostrale*) of this undemanding species need to be considered, sheep are a relevant preclinical animal model in the field of cerebrovascular diseases and may contribute significantly to translate different approaches into clinic research.

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Medial temporal lobe atrophy in poststroke cognitive impairment: 2 years follow-up study

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Abstract

Introduction: There are few longitudinal studies with controversial results examining delayed changes in cognition after ischemic stroke and predictive values of neuropsychological and neuroimaging markers. **Aim:** The objectives of this study were to evaluate the delayed change in cognition in poststroke patients and relationship to the medial temporal lobe (MTL) atrophy.

Material and Methods: Eighty-five first-ever stroke inpatients (mean age 65.6±5.6) without previous cognitive complaints were prospectively evaluated with a comprehensive neuropsychological battery on the 5-th day, 1-st, 6-th, 12-th, and 24-th month. A wide range of clinical, radiological and neuropsychological variables were examined.

Results: Our results showed that among all neuropsychological measures, only the IST test at the 2-year follow-up showed significant decline reaching the baseline level impairments in comparison with the results at 12-th month. On the other hand, MTL atrophy showed its high impact on the performance on all neuropsychological tests, even after 2 years post stroke. In addition, the MTL atrophy increased significantly two years after stroke compared with the baseline.

Conclusion: These results enriched our previous neuropsychological and neuroimaging longitudinal findings after stroke. Moreover, the data strengthened the important role of the initial assessment of MTL atrophy, and the follow-up IST examinations as a neuropsychological quick, easy and reliable tool for delayed poststroke cognitive impairment.

Key words: poststroke dementia; neuropsychological predictors; neuroimaging; hippocampal atrophy; IST

Introduction

Poststroke cognitive impairment is frequent. In community-based studies, the prevalence of poststroke dementia (PSD) is about 30% and the incidence of new onset dementia after stroke increases from 7% after one year to 48% after 25 years. [3] Some studies indicate that PSD may be reversible in a substantial proportion of patients.[6] By contrast, other studies found that not only the risk of PSD is high immediately after stroke but also it remains higher in patients non-demented three months after the stroke. There is a need to identify individuals at particular risk of poststroke cognitive decline and hence to target interventions for secondary prevention and improve the outcome and quality of life of stroke patients. Identifying a quick neuropsychological screening tool which could predict delayed cognitive and functional decline in stroke patients is a challenge. The role of neuroimaging markers, including white matter changes and medial temporal lobe atrophy (MTLA), as a contributor to poststroke cognitive decline is still controversial.[2]

We present results from a prospective hospital-based study of consecutive, first-ever stroke patients, designed to evaluate the delayed changes in cognition and their relationship to neuropsychological and neuroimaging markers. The study aimed to determine a quick cognitive screening test and neuroimaging markers measured in the acute poststroke phase which can accurately predict delayed cognitive and functional decline in poststroke patients.

Material and Methods

Eighty-five consecutive inpatients, aged 50–80 years, were included in this study, within three days of the onset of their first-ever ischemic cerebral stroke. Patients were recruited from the acute stroke unit of the Clinic of Neurology, Ruse, Bulgaria. Inclusion criteria for the study were: well documented clinical presentation and CT scan of the brain. Exclusion criteria included: 1) patients with a primary hemorrhagic stroke (intracerebral or subarachnoid); 2) patients with National Institutes of Health Stroke Scale (NIHSS) score > 6; 3) persistent aphasia; 4) history of pre-stroke cognitive impairment. We composed a control group of 25 normal control subjects (NCs), matched to the patients' group according to their age and educational level.

Assessments and data collection - Neurological and neuropsychological assessments were conducted at the baseline (5-th day after stroke onset), 1-st, 6-th, 12-th and 24-th month. Assessment of neurological status was determined using National Institutes of Health Stroke Scale (NIHSS) score.

Neuroimaging characteristics - A non-contrast CT brain scan examination was carried out for all patients on admission to the hospital and for 22 patients - at the 24-th month. For hippocampal rating, we used the scale described in detail by Scheltens et al.[4] It is a five point scale of hippocampal atrophy (normal=0, severe atrophy=4). The scale of Scheltens[5] (0-to-6-point), which evaluates the presence and extent of periventricular changes (PVCs) and deep white matter changes (DWMCs) in different anatomic regions, was used for PVCs and DWMCs. Wattjes et al. [7] described excellent intraobserver agreement between CT and magnetic resonance imaging (MRI) for medial temporal atrophy and substantial overall agreement concerning white matter changes with published visual rating scales.

Cognitive and neuropsychiatry assessments - A set of standardized neuropsychological tests, sensitive for mild cognitive impairment and covering major cognitive domains, were selected. As a substantial part of the Executive functioning, we assessed Cognitive flexibility using Trail Making Test Part A and B (TMT-A, B) and Verbal Fluency/Set sifting using Isaac's Set Test (IST), 15-seconds version. The IST assessed verbal fluency by measuring the ability to generate lists of words in four semantic categories (fruits, colours, cities and animals) in a 15 seconds interval.[1] The IST is considered to be a brief screening as a timed cognitive test with highly loaded executive functioning (cognitive set shifting, generation and processing speed), semantic and working memory, found to have predictive values for pre-clinical detection of dementia symptoms.

Results

The demographic data of the patients and the control group is presented in Table 1. The distribution of stroke lesions showed left middle cerebral artery (MCA) lesions in 29 subjects, right MCA lesions in 39 subjects and vertebrobasilar lesions in 17 subjects.

Table 1. Baseline characteristics of patients and normal controls (NCs)

BASELINE	NCs N=25	Poststroke patients N=85
Age	64.1 (5.3)	65.6 (5.6)
Gender (male/female)	18/7	67/18
Education	11.7 (2.2)	11.3 (2.2)
MMSE	27.9 (1.4)	26.6 (2.3) ***
Diabetes	4%	28% *
Hypertension	76%	86%
NIHSS	0	4.6 (0.9)

IST - Isaac's Set Test; MMSE - Mini Mental State Examination; NIHSS- National Institutes of Health Stroke Scale

Stroke patients had significantly lower performance on most of the cognitive tasks. The most significant differences were found on the tasks assessing orientation, executive functions and memory. General cognitive functioning (MMSE) score at baseline assessment was significantly below the mean score of NCs.

Patients had significantly lower scores on all measures of verbal learning. Their performance was characterized by reduced short-term memory, slow learning and lowered level of recollection from long-term memory. The pattern of memory impairment resembled the one observed in medial temporal lobe dysfunction. The most significantly impaired were all timed on tests of executive functioning – IST and TMT (A and B). All patients at baseline were cognitively slow, had non-effective set shifting and reduced semantic generation.

Among all neuropsychological measures, only Isaac`s Set Test at 2-nd year follow-up showed significant decline reaching the baseline level impairments in comparison with the results at 12-th month (Fig.1). The executive functioning deficit (cognitive speed and shifting) appears to be the most affected cognitive domain 2 years after stroke (Fig.2).

Fig.1. IST score after 24 months was back at the baseline (28 points)

Fig.2. The TMT-B value improve to the 12-th month and the 24-th month check showed a negative change

On the other hand, MTL atrophy showed high impact on the performance during attention/executive tests, even 2 years after stroke. In addition, the MTL atrophy increased significantly in that time compared with the baseline (Fig.3). Although CT is less accurate than MRI in demonstrating the degree of DWMCs and PVCs, a previous study showed an agreement on the grading of changes on this scale between CT and MRI[7].

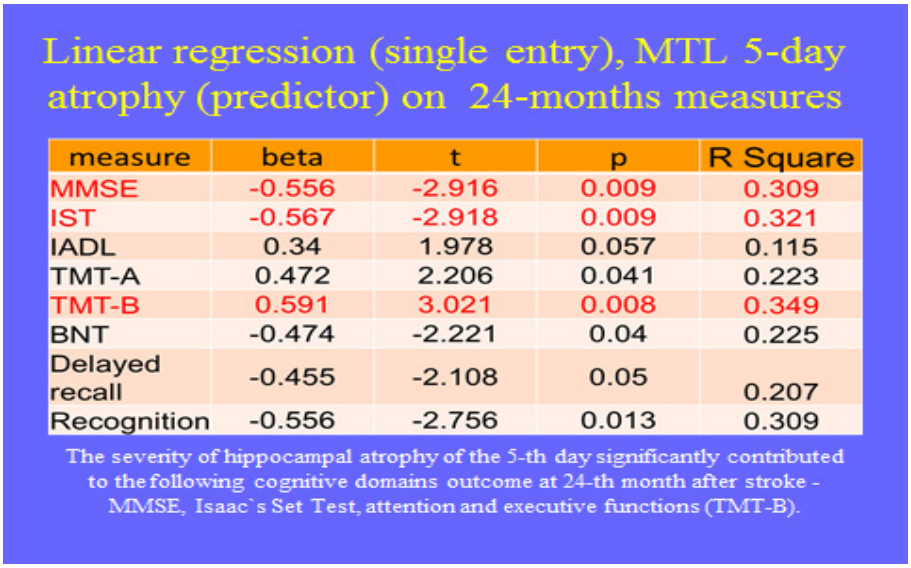

Fig.3. MTLA predictor of cognitive decline 2 years after stroke

Conclusion

These results underlined the important role of the initial assessment of MTL atrophy. In our study the hippocampal atrophy assessed by a visual scale is the strongest predictor of cognitive impairment and

dementia outcome, even in poststroke patients. The follow-up IST examination was a quick, easy and reliable neuropsychological tool for delayed poststroke cognitive impairment.

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The Effect of Computer-based Attention Training on Divided Attention Regarding to Age of Patients after Stroke

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Abstract

In the present study we compared the effects of computer-based cognitive rehabilitation of attention regarding to age of patients after stroke. The study included 16 subjects after first stroke treated as inpatients or outpatients at our Institute, who were included in intensive selective attention training four times a week for three months. Each patient was assessed at the initial and the final rehabilitation stage with the Divided attention task (TAP system; Test of Attentional Performance). The patients were divided in two groups regarding to age (Group 1: 8 patients younger than 45 years; Group 2: 8 patients older than 45 years). The results based on a mixed design ANOVA with one between subject variable indicate a significant moderate increase in performance on the divided attention task in both groups, while the interaction between change and age is not significant. We concluded that an intensive cognitive rehabilitation of attention in patients after stroke should start as soon as possible after the onset of the disease for majority of patients regardless of age.

Introduction

Deficits of the attention system after stroke are the most common and prominent consequences at the cognitive level of functioning (from 42% to 93% of all cases; Barker-Collo, *et al.,* 2009; Planton *et al.*, 2012). Due to its core functioning, the attention system is connected with all levels of cognition and therefore deficits can significantly decrease functional ability. In an extensive retrospective study Hurford et al. (2013) showed that information processing and maintaining of attention are the most frequently impaired cognitive systems, while being at the same time most prone to spontaneous recovery. The attention system can be rehabilitated cognitively mostly by training of the reaction speed and by learning the awareness and selection of suitable stimuli by means of computer-based paradigms. Cicerone et al. (2011) in their survey of the various types of cognitive rehabilitation found that the results of the attention training are transferrable and most successful when the training is aimed at higher levels of attention, namely selective and divided attention system levels.

The aim of our pilot study was to compare the effects of computer-based cognitive rehabilitation of attention regarding to the age of patients after stroke.

Materials and Methods

Subjects: The study included 15 subjects after first stroke, treated as inpatients or outpatients at our Institute, who were included in intensive selective attention training. The patients were divided in two groups regarding to age (Group 1: 7 patients younger than 45 years; $M_{(age)}$=32,5; $SD_{(age)}$=10,3; $M_{(onset\ of\ stroke)}$=5 months; $SD_{(onset\ of\ stoke)}$=4.7); Group 2: 8 patients older than 45 years; $M_{(age)}$=52,5; $SD_{(age)}$=3,6; $M_{(onset\ of\ stroke)}$=3 months; $SD_{(onset\ of\ stoke)}$=4.9).

The inclusion criteria for participating in the study were, that patients were without previous cerebrovascular diseases or other chronic degenerative disease, without severe motor impairments or speech and language disorders, without clinical signs of psychopathology or history of mental disease.

Instruments: Each patient was assessed at the initial and the final rehabilitation stage with the Divided attention task (TAP system; Test of Attentional Performance). For the purpose of training selective and divided attention the task Selective attention – Cross-modal on the rehabilitation software modules for computer-assisted cognitive rehabilitation CogniPlus was used. In this task the relevant and irrelevant stimuli (visual and auditory) suddenly emerge from the darkness. The subject is to respond only when relevant stimulus combinations emerge.

Based on the previously assessed patient's ability at the beginning of the training, the difficulty level (1 to 15) and the duration of the task (10 to 30 minutes) were selected by the therapist.

Methods: Patients participated in the intensive training of the selective attention module four times weekly for 30 minutes daily. During the training the therapist was present. At the end of the session the patients were educated about their effectiveness based on the program performance analysis. The patients were assessed by a TAP test at admission and three months later at discharge.

Results

The results of the 2x2 variance analysis show a significant increase in the efficiency of the divided attention ($F(1,13)=12.990$, $P=0.003$, $\eta^2=0.500$) in both groups, while the interaction between age groups and training does not reach the level of statistical significance ($F(1,13)=0.261$, $P=0.618$, $\eta^2=0.020$). The mean values for both groups are presented in Figure 1.

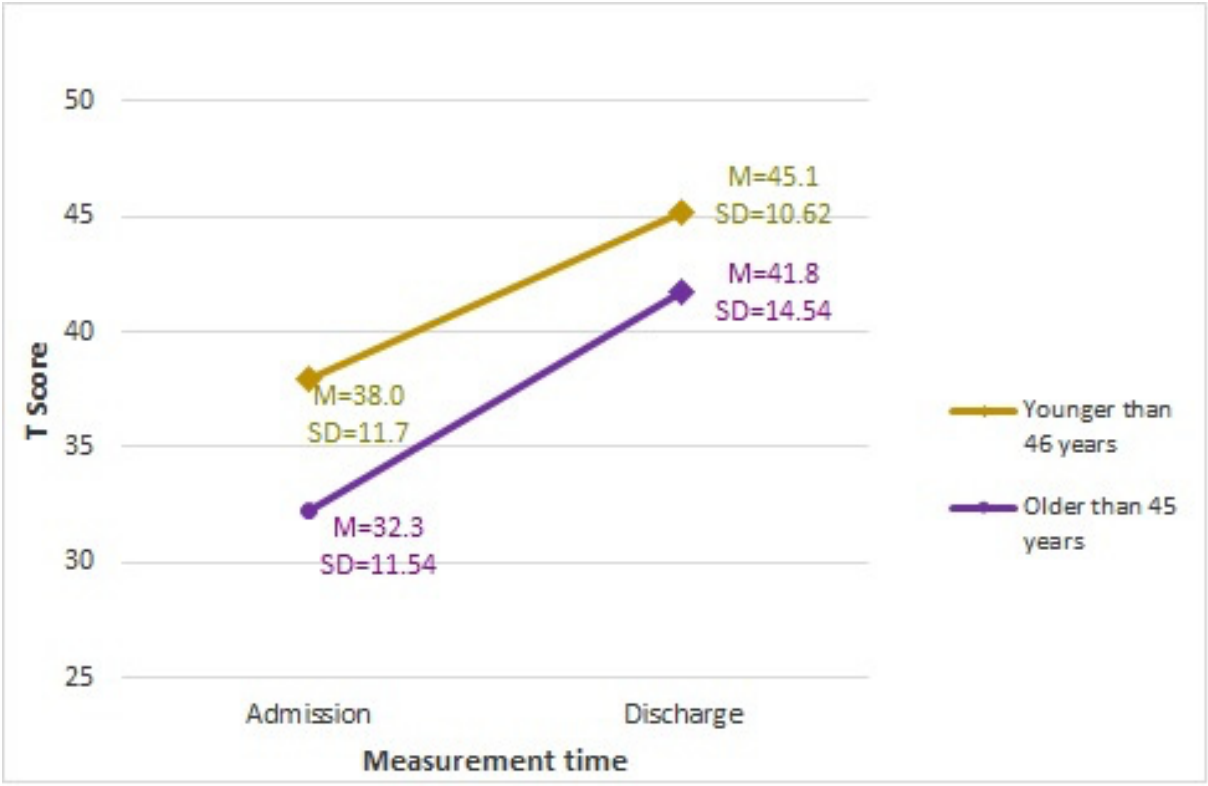

Figure. 1: Mean differences across groups of patients on the TAP Divided Attention test.

Conclusion

In this preliminary study we evaluated the effects of a three-month computer based intensive training of selective and divided attention in patients after stroke on the divided attention task regarding to patient's age. The results have shown moderate improvement on the divided attention test for both groups.

At the initial stage the group of younger patients indicated slightly higher scores on the task at admission and also at discharge compared to the group of older patients. Contrary the group of older patients indicated lower values at admission and also at discharge, but for both groups the increase in task results were similar, indicating similar improvement potential. Therefore we concluded that intensive cognitive rehabilitation of attention in patients after stroke should start as soon as possible after the onset of the disease for the majority of patients regardless of age. The computer-based paradigm aimed at training of the reaction speed and by learning the awareness and selection of suitable stimuli can be due to its ease of use effective for rehabilitation of older patients who may not have been accustomed to the use of a computer before the disease.

Although the effect of treatment is supported, research on larger samples is required.

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Obesity-related to type-2 diabetes and brain cholinergic dysfunction: preliminary evidence from Obese Zucker Rats

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Abstract

Obesity is associated with glucose intolerance, type 2 diabetes, and dyslipidemia. Type-1 and type-2 diabetes are related with reduced performance on cognitive function likely depending by changes of the cholinergic system. The obese Zucker rats(OZRs), with leptin receptors mutation, represent a model of obesity related to type-2 diabetes. This study has investigated cholinergic system of OZRs compared with non-obese cohort lean Zucker rats(LZRs) to assess possible relationships between obesity and brain disorder.

Male OZRs and LZRs of 16 weeks of age were used. Behavioural tests were performed to identify cognitive changes. Body weight, blood pressure and blood parameters were checked. The brain was processed for immunochemical and immunohistochemical analysis of neuronal specific nuclear protein (Neu-N), vesicular acetylcholine transporter (VAChT) and nicotinic(nAChR□7) were also evaluated.

OZRs of different ages, showed body weight, systolic pressure, glycemia, insulin, triglycerides and cholesterol levels higher in comparison with LZRs. Behavioural tests revealed in OZRs non changes in anxiety and emotional learning tasks.

In frontal cortex, morphological and immunochemical analysis revealed a decrease of Neu-N in OZRs compared to LZRs. In OZRs, a decrease of VAChT and nAChR□7 immunoreaction was observed.

These results may represent the first step to characterize neurological and cholinergic changes potentially occurring in brain of obese rats. This preclinical evidence may be useful to clarify the pathophysiology of brain damage reported in obese individuals.

Key words: Obesity, type-2 diabetes mellitus, brain cholinergic system.

Introduction

According to the World Health Organization (WHO), obesity is classified as having a total body fat percentage greater than 35% in women and over 25% in men [1]. The prevalence of obesity has increased dramatically worldwide over the last decades and has now reached epidemic proportions [2].

High body mass index is associated with the development of cardiovascular risk factors such as hypertension, dyslipidemia, insulin resistance and diabetes mellitus [3,4].

The presence of the above factors identifies metabolic syndrome (MetS), defined by several interconnected physiological, biochemical, and metabolic factors related to obesity and to the development of cardiovascular and cerebrovascular diseases [5].

Recent studies indicated that different caloric intake may influence neuronal function. Excessive caloric intake, associated with accelerated aging of the brain, rises the risk of neurodegenerative disorders [6]. Brain areas particularly vulnerable to obesity-related atrophy include the white matter of hippocampus, cingulate gyrus, and frontal lobes [7]. A body mass index (BMI) of 30 or higher has been linked with a decline in executive function over a ten-year period.

Obesity and type 2 diabetes mellitus (T2DM) are two interlinked conditions that have been attributed to brain atrophy. Recent studies indicate that T2DM significantly reduces the volume of the hippocampus and other brain structures [8]. An increased size of the temporal horn of the brain ventricles can be used as an indirect measure of atrophy in the hippocampus.

Obesity and Mets represent risk factors for adult-onset dementia disorders such as Alzheimer's disease (AD) and vascular dementia (VaD) [6]. They have been also associated with poorer cognitive performance in

population-based investigations. Moreover, combination of obesity and arterial hypertension could impair performance across various cognitive domains [9].

The cholinergic system is altered in age-related neurodegenerative diseases, such as AD and VaD a and is vulnerable to the effects of aging [10-12]. A decline in the integrity of the cholinergic system characterized, by a decrease of acethylcholine (ACh) biosynthetic enzyme choline acetyltransferase (ChAT), and by a reduction of cerebrospinal fluid ACh levels, was also reported in patients with VaD.

The purpose of the present study was to investigate brain microanatomy and cholinergic pattern, in Obese Zucker rats (OZRs) compared with their littermate lean controls Zucker rats (LZRs) at different ages. This to clarify the possible relationships between Mets, cerebral injury and cholinergic system impairment.

Materials and methods

2.1 Animals and tissue treatment

Male obese OZRs and the littermate LZRs of 16 weeks of age were used. The OZRs, with a mutation in leptin receptor, represent a model of T2DM exhibiting a moderate degree of arterial hypertension and increased oxidative stress [13]. LZRs, referred to as the dominant feature (*Fa/Fa*) and the characteristic OZRs, which actually has a recessive trait (*fa/fa*) of the leptin receptor, which can weigh up to 1 kg-more than double the average weight. OZRs were largely used for investigating mechanisms and pathophysiology of the disease including pharmacological treatment of it. Moreover, OZRs were considered a model for assessing the influence of MetS in the brain and the correlation with degenerative phenomena in obesity [13,14].

Body weight, food intake and blood pressure were first the sacrifice. In the blood samples levels of glucose, insulin, triglycerides and total cholesterol were measured using commercial kits. Behavioural tests (open-field and passive-avoidance) were performed to identify possible cognitive changes. After the sacrifice, the brain was dissected out and divided into two halves: the right hemisphere was used for performing immunochemical analysis, the left one was fixed and used for immunohistochemistry analysis.

2.2 Immunohistochemistry

Consecutive sections (10 μm) were processed for immunohistochemical detection of different protein using specific antibody:

- Neuronal specific nuclear protein (Neu-N) for identification of neuron;
- Vesicular acethylcholine transporters (VAChT), □7-nicotinic acethylcholine receptors (nAChR-□□7) as cholinergic markes.

After incubation with a specific biotinilated secondary antibody the product of immunoreaction was revealed by indirect avidin-biotin immunohistochemistry using diaminobenzidine as a chromogen. Sections were viewed under a light microscope. The images were transferred from the microscope to the screen of an IAS 2000 image analyzer for morphological and densitometric analysis.

2.3 Western Blot Analysis

Samples of frontal cortex and hippocampus were homogenized. Equal amounts of protein were separated by SDS-page and transferred to nitrocellulose membrane. Transblotted membranes were incubated with primary antibodies. Immunochemistry product was visualized using as HRP- biotinylated antibody followed by a chemiluminescence detection system, using a BIORAD Chemidoc XRS+ to acquire the images. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a protein reference.

Results

The value of body weight was significantly higher in OZRs compared to age-matched LZRs. Similarly, the food intake was higher in OZRs respect to LZRs indicating a condition of hyperphagia (Table 1). The values of systolic blood pressure were higher in OZRs. The values of glycaemia, insulin, total cholesterol and triglycerides were higher in OZRs compared to the LZRs (Table 1), indicating a condition of dismetabolism similarly to Mets.

Table 1. GENERAL AND BLOOD PARAMETERS OF LEAN ZUCKER (LZRS) AND OBESE ZUCKER (OZRS) RATS.

	LZRs	***OZRs***
Body weight (g)	356,1 ± 6.2	519,8 ± 7.8*
Food Intake (g/day)	24,4 ± 0.7	31,1 ± 0.65*
Blood Pressure mmHg	86,8 ± 9.93	105,8 ± 11.59*
Brain weight (g)	1,9 ± 0.04	1,7 ± 0.03*
Glicemia mg/dl	75.6 ± 5.1	94.1 ± 6.5*
Insulin ng/ml	0.8 ± 0.1	4.6 ± 0.9*
Triglycerides mg/dl	44.7 ± 3.8	312.7 ± 30.3*
Total Cholesterol mg/dl	73.2 ± 2.3	142.8 ± 5.63*

** = p < 0,05 vs LZRs*

The open-field test revealed the OZRs in all parameters a decrease of cumulative distance traveled, their number of rearings and increasing the total immobility time. On the other hand OZRs present a decrease in the number of entries into the central zone and the time spent by the animals in this area of the field compared the LZRs. In 16 week-old SHR no significant decrease of the ratio between time in zone 1 and central zone was observed. This indicates a condition of anxiety mediated by an increase of immobility time (data not shown). OZRs showed no evident reduction of retention latency time in the emotional learning task , such as passive avoidance test (data not shown).

Numerical evaluation of Neu-N positive neurons in the different layers of frontal cortex showed a different pattern in OZRs compared to LZRs, with layers VI and V displaying the most relevant changes. The number of Neu-N positive neurons in the VI layer, was decreased in 16-weeks-old OZRs (26.4 ± 3.9 n/0.05mm^2) compared to LZRs (39.7 ± 2.13 n/0,05mm^2 p <0.05*vs* OZRs) (Fig.1).

Immunoblots of frontal cortex for Neu-N revealed a decrease expression of the protein in the brain of OZRs compared to LZRs (Fig.1)

Western blot analysis, performed in the frontal cortex, showed a decreased expression of VAChT in 16-weeks-old OZRs compared to the age-matched controls LZRs (Fig.1). In cerebral cortex of LZRs rats, sparse nerve cell bodies polygonal in shape expressing in their external borders VAChT immunoreactivity were observed (Fig.1). These cell bodies were concentrated primarily in cerebrocortical layers V e VI. In OZRs, the network of VAChT-positive nerve fibers and cell bodies was less evident than in LZRs rats (Fig.1).

A reduced expression of nAChRα7 in frontal cortex and hippocampus was observed in 16-weeks-old OZRs compared to age-matched LZRs. Immunochemical evidence was confirmed by immunohistochemical analysis (Fig.1).

Conclusions

OZRs were developed as an animal model of obesity and T2DM. They were largely used for investigating mechanisms and pathophysiology of these diseases, including their pharmacological treatment. Moreover, OZRs were considered also a model for assessing the influence of Mets on in the brain and the correlation with neurodegenerative processes in obesity. The decrease of Neu-N positive neurons immunoreaction in OZRs, demonstrate a neurodegenerative process, can be related, among other causes, to the increase of systolic blood pressure values.

These results confirm the presence of VAChT in frontal cortex involved in cognitive functions with a reduction in 16-weeks-old OZRs. The reduction in the expression of nAChRα7 may be involved in the modulation of the inflammatory response at the central level. Actually, nAChRα7 may be involved in inflammatory processes and may improve sensitivity to insulin. The modulation of the cholinergic system in rats OZRs, it is an extremely complex phenomenon, and may be related both to inflammation induced by obesity and to cognitive impairment. The identification of neurodegenerative changes may represent the first step towards a better characterization of neuronal involvement in this animal model and possible treatments for countering it.

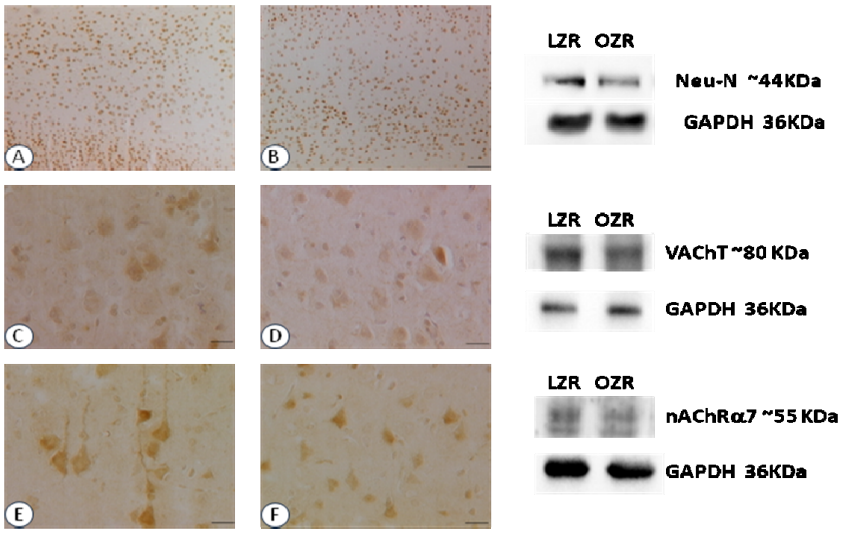

Figure 1. *Immunohistochemical detection of Neu-N (A,B) VAChT (C,D) and nAChRα7 (E,F) in the frontal cortex of lean Zucker rats (A,C,E) and Obese Zucker rats (B,D,F). The lane of the western blot were obtained using the same antibody. Calibration bar: A,B: 100 μm; C-F: 25 μm.*

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