Verbal fluency in research conducted with PET technique under conditions of extended cognitive activation with the use of 18F-fluorodeoxyglucose (FDG) tracer

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Summary

Introduction. Functional neuroimaging of the brain is a widely used method to study cognitive functions.

Aim. The aim of this study was to compare the activity of the brain during performance of the tasks of phonemic and semantic fluency with the paced-overt technique in terms of prolonged activation of the brain.

Methods. The study included 17 patients aged 20–40 years who were treated in the past for Hodgkin’s lymphoma, now in remission. Due to the type of task, the subjects were divided into two groups. Nine people performed the phonemic fluency task, and eight semantic. Due to the disease, all subjects were subject to neuropsychological diagnosis. The diagnosis of any cognitive impairment was an exclusion criterion. Neuroimaging was performed using PET technique with 18F-fluorodeoxyglucose (FDG) tracer.

Results. Performance of a verbal fluency test, regardless of the version of the task, was associated with greater activity of the left hemisphere of the brain. The most involved areas compared with other areas of key importance for the performance of verbal fluency tasks were frontal lobes. An increased activity of parietal structures was also shown.

Conclusions. The study did not reveal differences in brain activity depending on the type of task. Performing the test in both phonemic and semantic form for a long time, in terms

The study was not sponsored.
of increased cognitive control resulting from the test procedure, could result in significant advantage of prefrontal lobe activity in both types of tasks and made it impossible to observe the processes specific to each of them.

**Key words**: verbal fluency, PET neuroimaging, 18F-fluorodeoxyglucose (FDG)

**Introduction**

Owing to modern neuroimaging techniques, the progress of scientific research focused on searching for neural correlates of particular cognitive function measured with neuropsychological tests is observed. Direct observation of the brain function during neuropsychological testing lets the researchers to locate various active brain regions involved in performing cognitive tasks as well as better understand the mental operations underlying this performance. In addition, it allows the researchers to observe the specificity of symptoms according to the location of brain damage. Such an approach finds its application not only in scientific research, but also in neuropsychological diagnostics.

Functional brain neuroimaging is commonly applied in examination of speech and executive functions [1, 2]. Verbal fluency tests are often used to assess these functions. They measure the fluency of generating words beginning with a particular letter (phonemic fluency) or belonging to particular semantic category (categorical fluency), and the strategies of browsing through semantic memory storage [2–7].

In phonemic fluency tests the level of difficulty of generating words which depends on the first letter that is usually related to the frequency of the words beginning with this letter in frequency dictionary, is taken into consideration. In the test with F-A-S letter version the level of difficulty is increasing, while in alternative versions (C-F-L, P-R-W) it is decreasing. Suggested Polish equivalents do not retain the rule of monotonicity (e.g. K-P-S, K-P-M, K-O-P, K-O-S) [8, 9]. When conducting the test with only one letter, it is suggested to use the letter “K” [10].

What decide about the difficulty in the categorical fluency test are both the range and the colloquiality of categories. In the English version tests the common categories of a wide range, which refer to everyday life are e.g. animals, vegetables, fruit [3]. However, the test sometimes uses categories at varying levels of difficulty, in the Polish version of the test theses are e.g. animals and sharp objects [10].

The verbal fluency task, for the purpose of clinical diagnosis, is performed within a limited time period, usually one minute, regardless of the type of fluency and chosen letter or category [4, 5]. In research conditions it is not a strict rule because of technological requirements. The research is designed in such a way that the measurement possibilities could be fully used in particular neuroimaging techniques.

Theoretically, there are four ways to conduct the word fluency test. These are: 1) paced-overt; 2) paced-covert; 3) unpaced-overt; 4) unpaced-covert, where paced and unpaced refer to limited and unlimited testing time, and overt and covert refer to loud and quiet articulations.
The traditional approach, which depends on free production of words, is not optimal because of the uncontrolled time for response, whereas articulating words aloud may be risky due to generation of motor artifacts [6, 11]. However, researchers have stopped using “silent articulation” in order to avoid non-linguistic brain activation [2] resulting from the attention conflict and within motor speech system [12]. Other authors explain this phenomenon and refer to it as a response conflict [13] or inhibition [14], but the essence of the problem remains the same. Currently, tests with loud articulation of words (paced-overt and unpaced-overt) are considered to be the most appropriate [2, 11].

Previous results of research on letter and semantic fluency show that in both cases the brain region, where the neuronal activity is increased during the performance of tasks, is the large part of inferior left frontal gyrus (Brodmann areas 44 and 45) [11, 15, 16]. It is postulated that these areas determine the ability to generate the words and involvement of working memory in word fluency tests [11]. Additionally, the anterior cingulate cortex is the structure which is responsible for lexical selection. The research also confirms the role of a supplementary motor field and anterior cingulate cortex in the mechanism which is responsible for planning of response sequences and its inhibition during the performance of verbal fluency test [17, 18]. All mentioned brain regions may be associated with executive functions. In neuroimaging studies on verbal fluency the activation in the areas of left hemisphere associated with generation and articulation of words, such as inferior precentral gyrus and premotor cortex, is also observed [18]. In research conducted with PET on healthy people, Gourovitch et al. [19] showed that both letter and semantic tasks are related to the activity of anterior cingulate cortex, left prefrontal cortex areas, thalamus and cerebellum. It is postulated that cerebellum is involved in the regulation of motor speech functions, although the newest research shows that the right hemisphere of cerebellum has a particular influence on linguistic abilities [20]. However, these facts are based mainly on the analysis of dysfunction accompanying the damage of the cerebellum. Whereas the research conducted by Senhorini et al. [21] shows that during phonologic fluency test activation of cerebellum is higher in case of easier tasks than in case of more difficult tasks. In PET research conducted on healthy population, it was observed that letter fluency causes bigger activation of left prefrontal cortex, whereas categorical fluency of left temporal cortex. Also other studies show that letter and categorical fluency differ from each other to some extent in their basic mechanism, what is reflected in activation of different brain structures [2, 4, 6, 22]. For example, Baldo et al. [4] observed that semantic fluency depends on the activity of temporal cortex, and letter fluency depends on prefrontal cortex, whereas parietal cortex is involved in both cases. It has been noticed that brain structures located in the medial part of temporal lobe have the influence on the ability to generate the words based on the letter or semantic category, also by association with particular category (e.g. words associated with the word “car”). An important fact is that a major activation of medial part of temporal lobes, in par-
ticular hippocampus, is related only to categorical fluency, including the tasks which require the associations [23]. This suggests the participation of episodic memory in processing the semantic data. The result is compliant with the old references based on the examination of patients with selective damage to prefrontal and temporal cortex [24]. Lack of the activity in the medial temporal lobe (MTL) is not surprising considering the fact that it is proved that letter fluency depends more on the activity of frontal lobes, in precentral and inferior frontal gyrus which are a part of executive control system, than the categorical fluency [2]. Participation of episodic memory in tasks requiring generation of words in accordance with semantic criterion may be explained with role of associations in recalling the words which have idiosyncratic character [22]. An example of such a task may be an attempt of giving a list of words related to objects of everyday use which are at home. In this case, medial temporal structures, although underestimated, are really important. In some situations they play an auxiliary role towards the semantic memory. Similarly, Whitney et al. [23] noticed that there are significant differences between free and restrained test conditions of generating the words and proved that natural verbal fluency depends on the structure of hippocampus more than it has been stated before. They suggest that it is a completely new approach to linguistic processes organization considering also those brain areas which exceed traditional region around the lateral sulcus [23]. An additional evidence supporting those observations are results of the research on Alzheimer’s disease and semantic dementia indicating that the structure of hippocampus and frontal part of temporal lobes are responsible for categorical fluency impairment [25, 26].

**Aim**

The aim of this research was to compare the activation of particular brain areas during performance of letter and categorical verbal fluency task with paced-overt condition.

**Material**

The research encompassed 17 participants aged 20 to 40, who were treated in the past because of Hodgkin’s lymphoma, currently in remission state. The sample was recruited from among the persons involved in the standard control procedure for possible recurrence of the disease and the PET scanning is included in the normal course of this procedure. The decision to carry out research on persons previously treated for lymphoma, not individuals recruited from a population of healthy people, was due to the invasiveness of PET using FDG radioactive isotope. None of the participants reported recurrence of the disease. In addition, none of the people at the time of the study had symptoms of the disease. They were also no longer subjected to any treatment. Taking into account the possibility of the possible impact of chemotherapy on cognitive functioning in the adopted methodology the minimum
time after the last application of chemotherapy in those subjects have also been taken into consideration. According to the research reports, minimal interval since the last chemotherapy was 6 months [27].

In addition to medical criteria, excluding presence of lymphoma foci and symptoms, respondents also met the criteria of:

- intellectual norm (based on the result of the Raven’s Progressive Matrices Test);
- efficiency of cognitive processes within the age norms (based on results of Similarities, Digit Span, Symbol Digit subscales of the Wechsler Test which measure cognitive functions such as attention, working memory, ability to abstract and generalize);
- lack of serious mental and somatic diseases or brain injury (excluded in structured clinical interview).

All the participants fulfilled following criteria prior to their inclusion to the study.

The participants were divided into two groups in which experimental letter (9 respondents) or categorical (8 respondents) version of word fluency test was conducted. Participants were classified to the groups in accordance with homogeneity principle in respect of such demographic data as age and education. Because of the past disease of all the respondents the diagnosis of cognitive functions was conducted before classification to the examination.

The research was conducted in Oncology Center in Bydgoszcz. The examined persons gave an informed and voluntary consent for participation in the research. The research was permitted by the Bioethics Commission of Collegium Medicum in Bydgoszcz of the Nicolaus Copernicus University in Torun.

The respondents gave also clinical interview considering their somatic state and their psychical state was assessed with help of structured clinical questionnaire. Research method was based on the procedure used in the examination of functional neural correlates of general intelligence [28].

**Methods**

Performance of a task, which measures the verbal fluency, began 5 minutes after intravenous administration of glucose 5-7 MBq marked with fluoride 18 (18F) – FDG and lasted 20 minutes.

In the group performing the letter version, 20 arbitrarily chosen letters were given, regardless of the difficulty level (there were all letters of alphabet besides H, Ł, M, Z). In the group performing categorical version of the task, 20 arbitrarily chosen categories were used (animals, plants, vegetables, fruit, flowers, trees, clothes, countries, cities, food, drinks, insects, tools, occupations, instruments, colors, sports, household appliances, furniture, vehicles).

The respondents from both groups were asked to give as many words within 60 seconds for each criterion as possible, except of proper names. The brain examina-
tion began after 30 minutes since the tracer was administrated intravenously. PET examination was conducted in PET/CT scanner – Biograph mCT 128. The examined participants were lying still with opened eyes and uncovered ears. CNS acquisition was carried out for 10 minutes. CT scan, which is necessary to correct the attenuation, was conducted with “low dose” technique.

Statistical methods

The obtained results were compared with Standardized Uptake Value (SUV) using SCENIUM software normative database (Siemens Medical Solutions USA, Inc.). The analysis was made in two stages. In the first one, the level of glucose uptake in the areas significant for verbal fluency, i.e. frontal (with cingulate cortex), temporal, parietal and cerebellum areas was compared. In the second stage, more detailed comparisons of glucose metabolism in analyzed areas of brain structures were made. The researchers used the variance analysis with repeated measurements and quality predictors (the group performing the semantic fluency task vs. group performing phonemic fluency task). In order to check the assumption about sphericity, the Mauchly’s sphericity test was conducted. If the assumption had not been fulfilled, the corrections were made: Greenhouse-Geisser, Huynh-Feldt, and the lower-bound correction. In all cases, their appliance confirmed the results obtained using ANOVA. For post-hoc analysis, Sheffe’s test was used.

Results

First stage of the analysis, in which we compared the brain areas crucial for verbal fluency, showed significant differences in the scope of glucose uptake depending on the brain region (p < 0.001) and the hemisphere (p < 0.01). The type of the examination turned out to be irrelevant (p = 0.51). The highest level of glucose uptake was observed in the frontal area and lower level, not much but significantly, in parietal area (p < 0.001). Glucose uptake was visibly lower in temporal area and cerebellum (p < 0.001). The left hemisphere was much more active than the right one in frontal and parietal areas (p < 0.001), as well as in temporal areas (p < 0.05). In case of cerebellum (as a whole), no significant differences between hemispheres (p = 1.00) were observed (Figure 1).

In the frontal area the differences in glucose uptake (Figure 2) are related to the area (p < 0.001) and the hemisphere (p < 0.01) but not related to the type of task (p = 0.37). Additionally, differentiation of area activity turned out to depend on the hemisphere (p < 0.001). Particularly active in both hemispheres was lateral part of frontal medial gyrus (a). Moreover, half of the areas in the left hemisphere showed the activity similar to it (slightly lower, but statistically significant). These were: orbital part of frontal medial gyrus (b) (p = 1.00), supplementary motor field (p = 0.96), inferior frontal gyrus in triangular part (b) (p = 1.00), opercular part (a) (p = 8.84) and orbital part (c)
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(p = 0.65) as well as lateral part of superior frontal gyrus (b) (p = 0.45). In the right hemisphere the lateral part of frontal medial gyrus (a) was more active than the majority of other areas with exception of lateral part of superior frontal gyrus (b) (p = 0.88) and opercular part of inferior frontal gyrus (a) (p = 0.06).

When comparing the hemisphere activity, frontal area of left hemisphere (p < 0.01) was more active, although the post-hoc analysis revealed significant differences only in case of triangular (b) and orbital (c) part of inferior frontal gyrus (p < 0.001).

When it comes to parietal area, results of our research showed significant differences in glucose uptake depending on the area (p < 0.001) and hemisphere (p < 0.001) but not on type of the task (p = 0.75). Significantly higher glucose metabolism (than in other areas) was noticed in both hemispheres in the area of inferior parietal lobule and angular gyrus (p < 0.001). The area with lower glucose uptake in both hemispheres was superior parietal gyrus (p < 0.001). It can be stated that there is a higher activity of left parietal area than of a right one (p < 0.001). However, there are no such differences in case of inferior parietal lobule (p = 0.90) and supramarginal gyrus (p = 0.99).

Figure 1. Glucose uptake level in the areas crucial for word fluency in left and right hemisphere

Brain areas: Fr – frontal; Pa – parietal; Te – temporal; Ce – cerebellum
The activity of temporal area turned out to be differentiated by the area (p < 0.001) and hemisphere (p < 0.01). However, the differences related to the type of task were not identified (p = 0.56).

The most active area in temporal area in both hemispheres (Figure 4) was Heschl’s gyri. In their case the glucose metabolism was higher than in other temporal areas (p < 0.001). Temporal gyrus (superior, middle and interior) and fusiform gyrus in both hemispheres showed indirect i.e. lower activity than in Heschl’s gyri (p < 0.001) and at the same time higher than in other areas (p < 0.001). The least active were MTL, hippocampus, parahippocampal area, and temporal pole. Although the left hemisphere turned out to be more active than the right one (p < 0.01), post-hoc analysis revealed significantly higher glucose uptake in the left hemisphere only in the superior part of temporal pole (p < 0.05).

In case of cerebellum, it cannot be stated that there are general differences in glucose uptake related to left or right-side localization (p = 0.86). At the same time, the differ-
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Figure 3. Glucose uptake level in parietal area in left and right hemisphere

SPG – superior parietal gyrus; IPL – inferior parietal lobule; SG – supramarginal gyrus; AG – angular gyrus; PG – postcentral gyrus

ences in activity of particular areas of cerebellum, which are significant (p < 0.001), are dependent on the side (p < 0.001). Type of the task did not differentiate the level of activity (p = 0.41).

Cerebellum area, which is the most active from all areas in both hemispheres, is superior-middle-posterior part (cerebellum crus 6) (p < 0.001) except for cerebellum crus 2 in the left hemisphere (p < 0.01) and inferior-posterior cerebellum (cerebellum 7b) in the right hemisphere (p < 0.01). Moreover, in the right hemisphere of cerebellum the peduncle (cerebellum crus 4_5) and inferior-posterior cerebellum (cerebellum crus 7b) show the high glucose metabolism in comparison to other areas (at the same time right-sided glucose metabolism in this area was significantly higher than left-sided: p < 0.001). In the left hemisphere, the areas with higher glucose uptake were: cerebel-

lum crus 2 (with significantly higher left-sided glucose metabolism than right-sided: p < 0.001) and inferior-frontal-middle cerebellum (cerebellum crus 1). The lowest
STG – superior temporal gyrus; MTG – middle temporal gyrus; ITG – inferior temporal gyrus; MTL – mesial temporal lobe; H – hippocampus; PH – parahippocampal; HG – Heschl’s gyri; TPs – temporal pole: superior; TPm – temporal pole: middle; FG – fusiform gyrus

activity, i.e. significantly lower than in other areas, was noted in cerebellum crus 10 (p < 0.001) which is presented in Figure 5.

Discussion

Based on the obtained results it may be stated that performance of tasks requiring the verbal fluency processes, regardless of test version, is associated to a greater extent with the activity of left hemisphere of the brain. Frontal lobes belong to the most involved areas in performance of word fluency tests in comparison to other areas crucial for performance of those tasks. The highest activity was observed bilaterally in: superior (b) and middle frontal gyrus (a, b) and inferior frontal gyrus (a, b, c). It is worth noticing that the activity of supplementary motor area (SMA), whose role in cognitive processes is not fully defined yet, is higher. Prior results of neuroimaging research [11, 15–18] also documented the superiority of left-sided brain activity during
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Figure 5. Glucose uptake level in cerebellum areas of right and left hemispheres

The performance of verbal fluency test and significantly higher activity of frontal lobes in comparison to other brain structures.

The researchers indicate also other structures, which are crucial for cognitive functions which are activated during performance of verbal fluency test, i.e.: parietal lobes [4], temporal lobes [29, 30] but also supplementary motor area [17] and cerebellum structures [19, 20].

Involvement of parietal areas in the performance of verbal fluency test is supported by results of Baldo et al. [4] and Gourovitch et al. [19]. At the same time this activity is similar regardless of the type of task. Moreover, posterior areas of parietal cortex are often associated with attention involved in repetition system which is a mechanism of memorizing the verbal material but also with processes of storing the verbal information in working memory [31].

Cerebellum for a long time was considered to be a centre which is responsible for coordination of movement and keeping of body balance. This is why its activity during cognitive tasks was earlier linked with motor aspects of speech processes, including the fluency of speaking. However, the research on cerebellum functions showed that it is also involved in complex cognitive processes [32, 33]. Functions such as planning, behavior control associated with inhibition of automatic reactions and the ability to switch the attention were earlier associated with prefrontal cortex areas [34], whereas it
turned out that also subcortical structures, including cerebellum, are involved in these processes [35]. Purkinje cells in the cerebellum mediate in comparing the intended and performed activity which enables to correct mistakes. Making a mistake activates Purkinje cells in order to correct it [36] in feedback loop mode which is an element of learning the new motor reaction.

Many studies indicate the role of temporal lobes, particularly in solving the categorical version of word fluency test. However, Gourovitch et al. [19] observed that the activity of temporal areas (as well as frontal areas) is significant for efficient performance of both versions of verbal fluency test. Some researchers claim that activation of temporal lobes during performance of cognitive tasks, including verbal fluency test, is caused by the fact that the respondents hear their own voices when giving an answer [37].

Similarly to mentioned literature, the results of this research confirm that performing the verbal fluency test is related to higher activity of some parietal structures (bilaterally: inferior parietal lobule and angular gyrus) and temporal structures (bilaterally: superior, middle, inferior temporal gyrus, Heschl’s gyri and fusiform gyrus). The results are also in agreement with previous findings about involvement of cerebellum in verbal fluency tasks, including the structures of cerebellum crus 7b bilaterally and peduncle in the left cerebellum (cerebellum crus 4_5) and also structures of right cerebellum crus 1 and cerebellum crus 2.

In the literature, the data acknowledge that dorsolateral prefrontal cortex (with middle frontal gyrus), inferior frontal gyrus and SMA, and also cerebellum are the areas which are usually related to processes of working memory and executive functions. There is experimental evidence that frontal lobes activity, in particular left middle frontal gyrus is associated with initiating the reaction in accordance with the task purpose, which in the verbal fluency test is generation of words in compliance with particular criteria [38, 39]. At the same time the literature emphasize the function of dorsolateral prefrontal cortex (DLPFC) [40, 41] executing the choice of reaction and allocation of attention resources on stimuli in accordance with undertaken purpose, and also the involvement of DLPFC in working memory processes which enables to keep in mind the information on rules and purpose of performed task [40]. Activation of inferior frontal gyrus and SMA during performance of some cognitive tasks [17, 42, 43] is associated with the engagement of these structures in processes of choosing the appropriate reaction, so the selection based on criterion [44, 45] including also inhibition of the reactions which are not compliant with determined criterion. Their involvement in performance of verbal fluency test is confirmed by results of this research, as well as by prior findings [16, 17, 46].

However, the results of this research did not confirm the activity in anterior cingulate cortex during performance of word fluency test. In Cohen’s model [47] this brain area controls the risk of mistake and cognitive conflict, and then it sends the information to DLPFC which, thanks to its high activity, indicates the intense executive control. We are assuming that in both examined groups the procedure and examination condi-
tions might have an influence on higher need of executive control which was taken over by the prefrontal cortex.

The fact that there are no differences of brain activity during the performance of phonemic and semantic versions of word fluency test is contradictory with some earlier observations [2–7]. According to the previous findings, basic difference in the activity is related to higher involvement of frontal lobes in performance of phonemic version and temporal lobes in performance of semantic version of verbal fluency test [4, 39, 48]. However, there are data indicating that patients with frontal lesions have similar low level of performance both in phonemic and semantic version of the verbal fluency test [49, 50]. It may indicate the greater impact of frontal lobes on the verbal fluency test performance, regardless of its version. Bigger activity of prefrontal regions in the examined group could result from the research procedure. The participants were examined on an empty stomach and were waiting for information about their health – a possible relapse of cancer. Both factors might be the reason for distraction, which increased the need of higher level of cognitive control. So in both versions of the word fluency tests the highest activity of prefrontal lobes associated with executive control, in comparison to other brain areas, but comparable for phonemic and semantic fluency, [47] was observed. There is also another possible explanation to the lack of difference between patterns of brain activity in phonemic and categorical version of the verbal fluency test which probably results from the testing procedure. It required a 20-minute, so relatively long and monotonous, cognitive activity of words generation which might cause the appliance of various, not only specific for each task, strategies of browsing the memory storage.

Certain limitation of this work, suggesting caution in the interpretation of the results is the fact that the study group consisted of people treated in the past due to Hodgkin’s lymphoma. As it has been pointed out earlier, all subjects were in remission confirmed in a control PET study and were no longer in treatment (they were at least 6 months after the last chemotherapy). According to the literature [27] after 6 months no effect of chemotherapy on cognitive functioning was observed. In addition, the inclusion criteria included the diagnosis of cognitive and intellectual functions. Another limitation was a small number of subjects and the lack of a healthy control group. We are aware that a greater number of patients would increase the likelihood of generalization on the basis of the obtained data. On the other hand, the international literature provides the results of neuroimaging studies conducted in samples of similar size [51, 52] as in the study conducted by our team. Additionally, the lack of control group was compensated by the use of SCENIUM software normative database which may be justified for ethical reasons. In this way, we avoided the unnecessary exposure of healthy controls on radioisotope, and participants in the experimental groups were subjected to the control medical procedures including PET. Despite these limitations, the results of this study provide a valuable attempt to check the effectiveness of PET technique in the study of verbal fluency in conditions of prolonged activation of the brain. They also point to the need for further
research into the characteristics and distinct mechanisms underlying phonemic and semantic fluency.

**Conclusions**

To summarize, it can be stated that verbal fluency test, regardless of version, causes the activation of structures which are often related to a wide range of executive functions. However, it is worth emphasizing that the verbal fluency test should not be considered as a tool sensible only to lesions or frontal dysfunctions because its performance is also related to activity of other cortical and subcortical structures. Our research revealed the highest activity in frontal structures (bilaterally: superior (b), middle (a) and inferior frontal gyrus (a) and left: middle (b) and inferior (b, c) frontal gyrus and supplementary motor area). Moreover, higher activity was found also in parietal structures (bilaterally inferior parietal lobule and angular gyrus). Less active were temporal areas and cerebellum. However, also in this case the research indentified the areas of higher activation (for temporal areas: bilaterally Heschl’s gyri, superior, middle, inferior temporal gyrus, and fusiform gyrus; for cerebellum: bilaterally cerebellum crus6, right: cerebellum crus 7b, peduncle (cerebellum crus 4_5), and left: cerebellum crus1 and cerebellum crus 2).

Conducted research did not reveal the differences in brain activity between phonemic and categorical version of verbal fluency test. It may result from the fact that performance of both tasks, in conditions which require increased cognitive control, might cause a dominance of prefrontal activity in both cases and disable the observations of processes specific to each version of the test.

**References**


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