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Short Communication

Detection of cytokine-induced sickness behavior after ischemic stroke by an optimized behavioral assessment battery

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ABSTRACT

Stroke causes severe and long-lasting symptoms in patients. Besides focal deficits such as speech impairment and limb weakness, stroke also results in neuropsychiatric symptoms, including fatigue, anxiety, and depression, which are debilitating and often impair post-stroke rehabilitation. However, in experimental stroke research, the study of neuropsychiatric symptoms and their therapeutic targeting has so far been largely neglected, which can be mainly attributed to the lack of appropriate tools to investigate such deficits in mice.

Here, we report that neuropsychiatric symptoms can be differentiated from focal deficits and specifically modulated independent of treating the primary lesion. In order to achieve this, we developed a novel behavior analysis tool by assessing test performance of various tests, combining outcome parameters to cover functional domains of focal and neuropsychiatric symptoms, and finally weighted results into a time point-specific score. This weighted score enabled us to clearly differentiate focal deficits and neuropsychiatric symptoms and detect these until the chronic phase after stroke. Using this analysis tool, we detected that neutralizing systemic cytokines (TNF-α, IL-1β and IL-6) specifically ameliorated neuropsychiatric symptoms but did not affect focal deficits or lesion volume. Hence, most conventional studies analyzing only focal deficits and lesion volume as primary outcome measures would have missed these significant and translationally relevant therapeutic effects. We anticipate that these findings will encourage more detailed analyses of neuropsychiatric symptoms particularly for anti-inflammatory therapies in stroke and that the presented weighted composite score will facilitate this development.

1. Introduction

Stroke is a major cause of mortality and disability in developed countries. Besides focal deficits such as speech impairment and limb weakness, stroke also results in neuropsychiatric symptoms that are debilitating and often impair post-stroke rehabilitation (Hackett et al., 2014; Robinson and Jorge, 2016). Common neuropsychiatric deficits after stroke include fatigue, sleep disorders, anxiety and depression (Hackett et al., 2014; Ferro et al., 2016); which can last from the acute phase after brain ischemia until the late chronic phase and substantially contribute to long-term morbidity of stroke patients. These neuropsychiatric symptoms are also commonly observed in the syndrome known as cytokine-induced sickness behavior (CISB) (Dantzer, 2001). CISB was first reported in the context of infections and is caused by the neuroactive function of certain pro-inflammatory cytokines (e.g. TNF-α, IL-1β, and IL-6).

Consequently, the inflammatory cause of CISB-related neuropsychiatric symptoms has been proposed in a large variety of diseases from infections to cancer and neurodegeneration (Kelley et al., 2003). Intriguingly, also an ischemic brain lesion results in a sterile, systemic immune response. We and others have previously demonstrated that soluble molecules released from the necrotic brain tissue after stroke can activate the peripheral innate immune system, causing the secretion of high levels of pro-inflammatory cytokines after stroke (Liesz et al., 2015; Roth et al., 2018; Denes et al., 2010). It is therefore likely that the systemic pro-inflammatory immune response after stroke contributes to the neuropsychiatric stroke symptoms independent of the primary lesion location in the brain. Indeed, we have previously demonstrated that blocking the acute pro-inflammatory response to stroke in the peripheral immune compartment improves signs of CISB in a mouse stroke model without affecting the primary lesion severity or focal deficits (Liesz et al., 2015). Hence, therapeutic targeting of the systemic immune

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response to stroke is a very promising, yet, vastly understudied approach to improve functional stroke outcome and quality of life for stroke patients.

A major obstacle for in-depth mechanistic studies and pre-clinical therapeutic studies targeting CISB after stroke is the lack of sensitive behavior tests differentiating between focal and neuropsychiatric stroke symptoms in the rodent stroke model. The use of behavior tests has become standard in most reports of preclinical stroke research; however, unbiased and multicenter assessments of their tests performance reveals that most of the commonly used tests have a very low sensitivity and do not cover neuropsychiatric deficits but focus on simple focal dysfunctions such as body asymmetry or limb weakness (Balkaya et al., 2013, 2018; Llovera et al., 2015; Rosell et al., 2013).

Therefore, we aimed to establish and test a comprehensive behavioral test battery. The novel weighted composite score established in this study, comprising 9 test parameters from six tests, covers focal deficits (motor, sensory, coordination) as well as various domains of cognitive function and neuropsychiatric symptoms. Using this novel tool, we could identify that neuropsychiatric symptoms are still present in the chronic phase (28d) after stroke and can be specifically modulated by cytokine-neutralizing antibodies independent of lesion severity and focal deficits.

2. Results

We selected six behavioral tests which cover the most common focal as well as neuropsychiatric deficits observed in rodent stroke models across various behavioral domains (Fig. 1**A**). All six tests were selected by their availability in most rodent laboratories and because they can be performed without large equipment. These tests were performed at baseline before stroke and up to 1 month after stroke induction by transient middle cerebral artery occlusion in order to analyze the individual test performance throughout the acute-to-chronic observation period and where compared to the performance of Sham-operated animals.

Focal deficits were assessed using cylinder test (motor), accelerating rotarod (coordination), and adhesive removal test (sensory, ART). The cylinder test exhibited prominent forelimb asymmetry during rearing movements up to 21d after stroke (Fig. 1**B**). The latency to fall from the accelerating rotarod was decreased throughout the complete 28d follow-up period post-stroke (Fig. 1**C**). ART detected a decreased ability to remove the adhesive within the first week after stroke but was less sensitive in the chronic phase (Fig. 1**D**, Suppl. Fig. 1A).

Neuropsychiatric symptoms (anxiety, activity, exploratory behavior, and anhedonia) after stroke were assessed by the elevated plus maze (EPM), open field (OF) test, and the sucrose consumption test (SCT). A decreased frequency of entering and reduced time spent in the open arms of the EPM was observed until 14d post-stroke as a potential sign of increased anxiety (Fig. 1**E**, Suppl. Fig. 1B). In the OF test, the overall distance moved per trial was decreased over the whole post-stroke period until 28d, indicating a general reduction in motivation or activity (Suppl. Fig. 1C). Furthermore, we observed that stroke-operated mice spent less time in the central area of the open field—an indicator of physiological, exploratory behavior—up to 14d after stroke compared to Sham-operated animals (Fig. 1**F**). Finally, we assessed signs of anhedonia, a key symptom of depressive-like behavior, by testing the normal preference of mice for sweetened water in the SCT (El Yacoubi et al., 2003). We detected that post-stroke mice consumed significantly less sucrose-water compared to sham-operated mice—losing their preference for sweetened water as a sign of anhedonia—during the first 14d after stroke but acquired again the preference for sucrose-water thereafter (Fig. 1**G**).

Commonly used behavior assessments after experimental stroke not only generally do not analyze neuropsychiatric symptoms, but also are not sensitive enough to detect long-term deficits in the chronic phase after stroke. In fact, most established behavior assessments—including those tested in this work—are not sufficiently sensitive to detect deficits beyond 14d post-stroke (Balkaya et al., 2013; Li et al., 2004; Manwani et al., 2011). Therefore, we aimed to establish a test battery based on the most sensitive readouts per behavior test across the various functional domains for each specific time point post-stroke. First, we performed a dimensionality reduction by principal component analysis (PCA) using a total of 9 parameters for each time point analyzed after stroke or sham surgery: cylinder test (forelimb asymmetry), rotarod (latency to fall), ART (sensing time), ART (removal time), open field (wall/open ratio), open field (distance), EPM (open/closed ratio), EPM (entering frequency), SCT (sucrose preference). The obtained data was then successfully tested for sufficient commonality, uniqueness, complexity and 5 additional parameters based on published guidelines for PCA analysis and reporting (Budaev, 2010) (Suppl. Tables 1 and 2). Moreover, an overview for the eigenvectors shows significant variance (PC1 $>$ 2; PC2 $>$ 1) within PC1 and 2 throughout all investigated timepoints (Suppl. Fig. 2). This analysis revealed that combined behavioral information (Fig. 1**H**), in contrast to single tests or the combination of only tests for focal or neuropsychiatric deficits (Suppl. Figs. 3 and 4), has the potential to differentiate between stroke and sham conditions even 28d after surgery.

Next, with the goal to determine the most relevant outcome parameters per time point contributing to the improved test sensitivity, we calculated the factor loading for the Top 5 parameters to the first principal component (PC1) per post-stroke time-point (Fig. 1**I**). This loading factor per outcome parameter was used to calculate a weighted composite score which takes into account the differential contribution of test parameters per time point (Fig. 1**J**). With this weighted composite score, statistically highly significant differences could be detected between sham and stroke groups even up to 28d post-stroke.

After establishing this novel and highly sensitive analysis tool for complex post-stroke behavioral deficits, we aimed to determine its potential to detect specific effects of a therapeutic approach on neuropsychiatric symptoms. To this end, we treated animals with neutralizing antibodies against IL-6, TNF- α and IL-1 β, the key cytokines reported to induce CISB, and determined the impact of this systemic anti-cytokine treatment on behavioral deficits (Fig. 2**A**). In accordance with previous reports, the systemic pro-inflammatory cytokine neutralization did not affect infarct volume (Fig. 2**B**). Correspondingly, behavioral tests for focal deficits also did not detect a significant difference between the control and cytokine neutralization groups (Suppl. Fig. 5A). Hence, most experimental stroke studies using these typical readout parameters (stroke volume and focal deficits) would not detect a therapeutic effect of the cytokine neutralization. However, utilizing the weighted composite score we detected a highly significant behavioral difference between the treatment groups despite similar infarct volumes (Fig. 2**C**). The ability to discriminate between treatment groups became even more apparent in the PCA, showing complete separation between all mice receiving either control or anti-cytokine treatment (Fig. 2**D**). As expected, this sensitive differentiation between treatment groups was based on the tests for neuropsychiatric symptoms included in the weighted composite score (Suppl. 5C-E). However, test performance of the weighted composite score still performed significantly better compared to the individual tests. This was revealed by receiver operating characteristic (ROC) curve analysis showing an excellent test performance with an area under the curve of 92%, which was higher than any of the individual tests (Fig. 2**E**).

Taken together, we report here a novel behavioral assessment tool that increases the sensitivity of conventional behavior tests. The increased sensitivity enables to detect behavioral deficits also in the chronic phase after stroke. Most importantly, the presented test battery and analysis method includes highly sensitive readouts for neuropsychiatric symptoms. Our results from a systemic cytokine neutralization experiment demonstrate that such neuropsychiatric symptoms are modulated independent of the primary lesion and can be clearly differentiated from focal deficits even in the mouse stroke model.

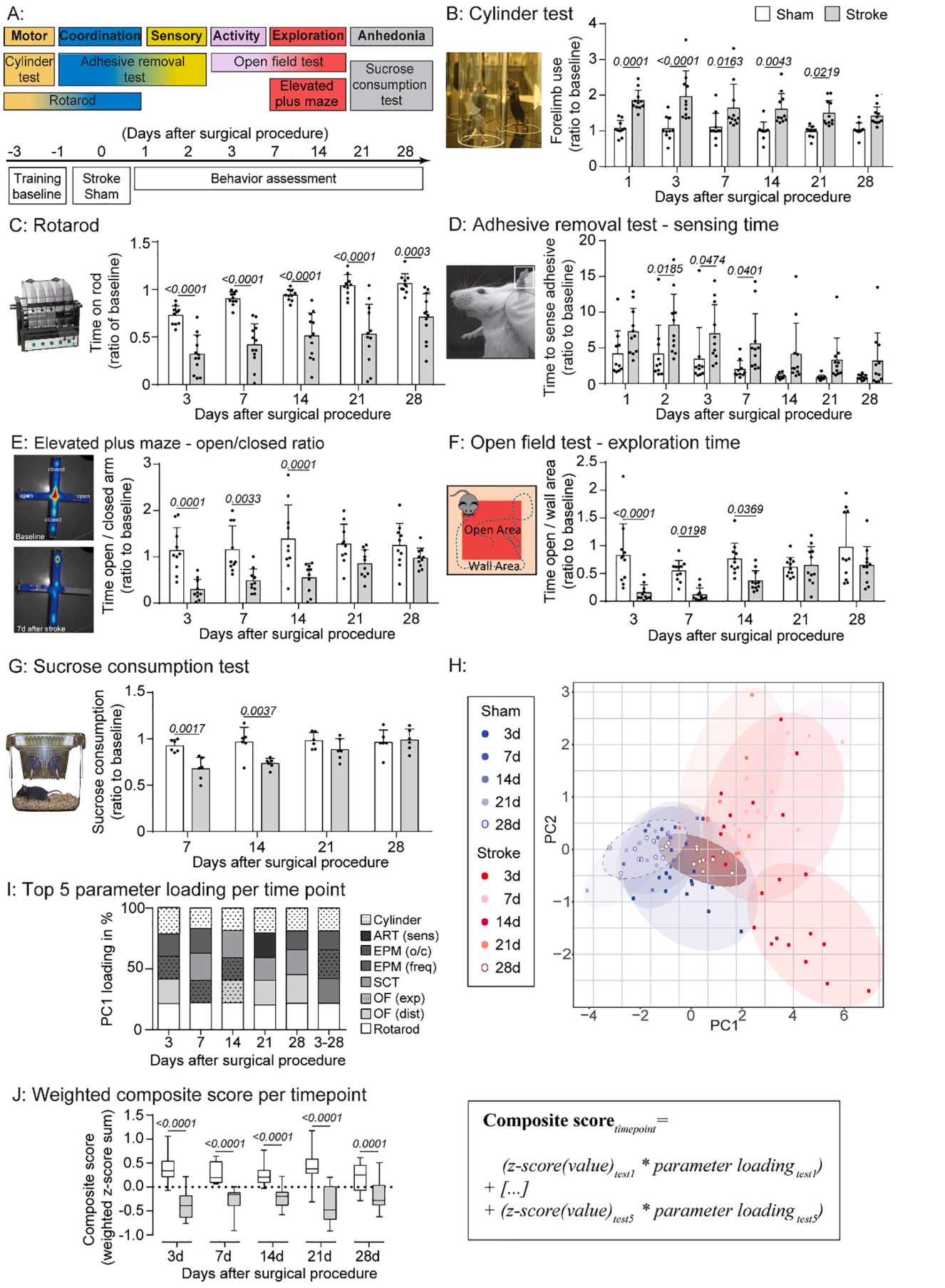

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Fig. 1. Development of a tool for assessing complex behavioral deficits post-stroke. (**A**) Schematic overview of the functional domains and associated individual tests as well as study design for acute-to-chronic behavior assessment after experimental stroke and sham surgery. (**B**) Quantification of cylinder test performance as forelimb use asymmetry up to 28d after surgery. Values are shown as intra-individual ratios to baseline. (**C**) Accelerating rotarod for assessment of motor and coordination deficits. Values are shown as percentage of time spent on rod normalized to baseline performance. (**D**) Quantification of time required to first sensing of the adhesive on the affected paw (contralateral to stroke hemisphere) in the adhesive removal test. Values are normalized to baseline of the same paw. (**E**) Elevated plus maze was performed to measure signs of anxiety. The open/closed arm preference was calculated as a ratio of time spent in open arms divided by time spent in closed arms and normalized for values post-stroke to the individual baseline, which was acquired 3d before sham or stroke surgery. (**F**) Quantification of exploration time in the open field test was calculated as time spent in the central area divided by time spent in the wall area. Post-stroke result was intra-individually normalized to baseline values. Statistical analyses in B-F have been performed by two-way ANOVA, n(stroke) = 11, n(sham) = 10. (**G**) Quantification of sucrose consumption, data is shown as the ratio of sucrose water / normal tap water normalized to baseline after stroke or sham surgery compared to baseline preference (Kruskal-Wallis Test, n = 6 per group). (**H**) Principal component analysis (PCA) for all test parameters obtained per time point. Areas for sham and stroke sample clusters at 28d after surgery are marked with a dashed line. (**I**) Loading of the top 5 test parameters contributing to the first principal component (PC1) for each time point after surgery. ART(sens) = adhesive removal test (sensing time); EPM(o/c) = elevated plus maze (open/closed arm preference); EPM(freq) = elevated plus maze (frequency of entering open arms); SCT = sucrose consumption test; OF(expl) = open field test (exploration: ratio of open/wall area); OF(dist) = open field test (distance moved). (**J**) A composite score was calculated based on weighted parameters from the time-dependent PC1 loading and weighted z-scores were summed for the respective top 5 parameters per time point. Right: formula for calculation of weighted composite score. Left: statistical analysis (H test) reveals significant differences detected until 28d post-stroke.

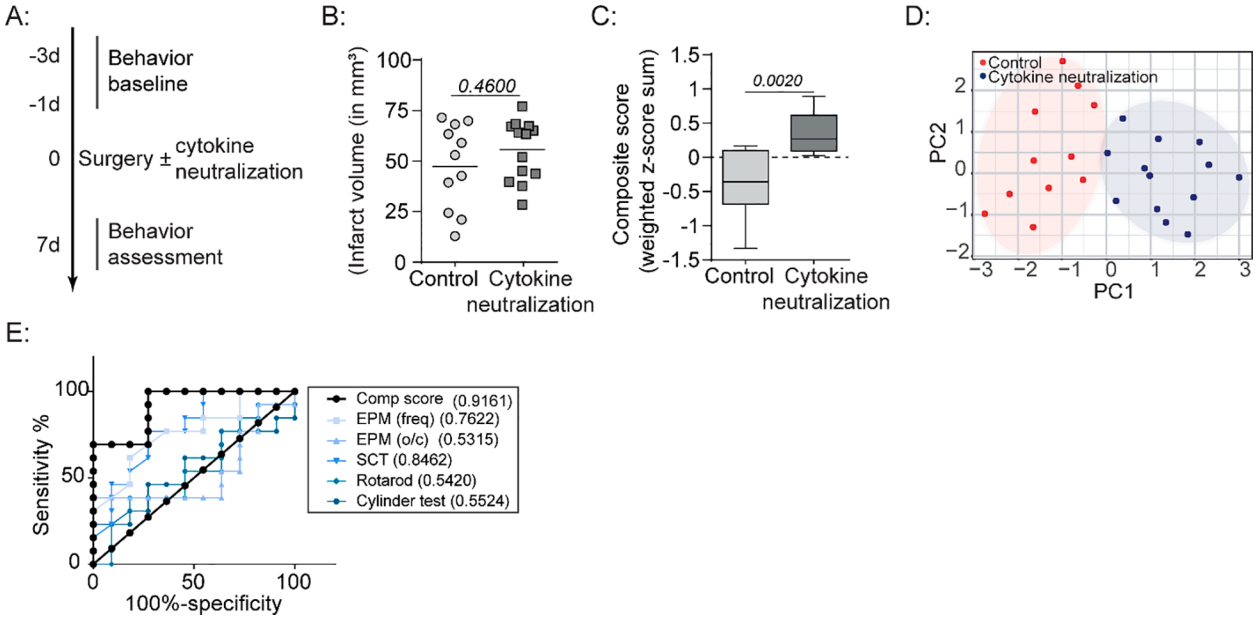

Fig. 2. Changes of neuropsychiatric CISB symptoms can be detected independent of primary lesion and focal deficits. (**A**) Schematic overview of the experimental design for cytokine neutralization (combined anti-IL-1 β, -IL-6 and -TNF- α) after stroke. (**B**) Pro-inflammatory cytokine neutralization does not affect stroke lesion volume. (**C**) Weighted composite score of elevated plus maze (open/close; frequency), sucrose consumption test, rotarod and cylinder test 7d after surgery (H-test). (**D**) Principal component analysis of behavioral assessments 7d after surgery. (**E**) Receiver operating characteristics (ROC) curve analysis of the weighted composite score compared to the performance of individual test parameters (values indicated as AUC = area under curve). All graphs: n(cytokine neutralization) = 13, n(control) = 11).

Systemic cytokine neutralization improved activity and exploratory behavior and reduced anxiety in post-stroke animals, despite no effect on primary infarct volume and focal deficits.

However, a limitation of the study was the usage of young, healthy mice. While most experimental stroke studies use young, healthy and male mice for practicality and homogeneity reasons, stroke occurs on average in elderly and comorbid patients of both sexes. Several studies have reported distinct features in aged versus young rodents after experimental stroke including differences in neuroinflammation, neurogenesis and tissue recovery (Buga et al., 2013; Popa-Wagner et al., 2014). Specifically, behavioral phenotype might significantly differ in aged mice as well as in animals bearing other common comorbidities of stroke patients and needs to be considered in future studies adopting the presented behavior test battery (Buga et al., 2013; Popa-Wagner et al., 2014).

Not a single clinical trial so far has been able to demonstrate significant reduction of infarct lesion or primary functional outcome to be improved by over 1,000 therapies that have been preclinically developed (O'Collins et al., 2006). While numerous suggestions have been made to overcome this translational roadblock (Begley and Ellis, 2012; Howells et al., 2014; Llovera and Liesz, 2016), an alternative approach could be to target therapies beyond tissue protection and focal deficits. Neuropsychiatric symptoms such as sleeping disorders, fatigue, malaise and depressive behavior are commonly observed in stroke patients (Dantzer, 2006; Crosby et al., 2012; Wallace et al., 2012) and they significantly affect secondary morbidity and physical recovery (Lerdal and Gay, 2013; Naess and Nyland, 2013; Willey et al., 2010). Yet, experimental as well as translational stroke research is barely studying mechanisms and therapeutic amelioration of these stroke symptoms. With the weighted composite score established and validated in this study, we anticipate to contribute a valuable tool to further promote experimental research on neuropsychiatric symptoms after stroke.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.11.016.

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