Research article

Soluble guanylate cyclase stimulator riociguat improves spatial memory in mice via peripheral mechanisms

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ABSTRACT

Soluble guanylate cyclase (sGC) - cyclic guanosine monophosphate (cGMP) signalling is important for healthy memory function and a healthy vascular system. Targeting sGC-cGMP signalling can therefore be a potential strategy to enhance memory processes. sGC can be targeted by using agonists, such as sGC stimulator riociguat. Therefore, this study aimed to target sGC using riociguat to investigate its acute effects on memory function and neuronal plasticity in mice. The effects of riociguat on long-term memory and a biperiden-induced memory deficit model for assessing short-term memory were tested in the object location task, and working memory was tested in the Y-maze continuous alternation task. Pharmacokinetic measurements were performed within brain tissue of mice, and hippocampal plasticity measures were assessed using western blotting. Acute oral administration with a low dose of 0.03 mg/kg riociguat was able to enhance working-, short-, and long-term spatial memory. Under cerebral vasoconstriction higher doses of riociguat were still effective on memory. Pharmacokinetic measurements revealed poor brain penetration of riociguat and its metabolite M₁. Increased activation of VASP was found, while no effects were found on other memory-related hippocampal plasticity measures. Memory enhancing effects of riociguat are most likely regulated by vascular peripheral effects on cGMP signalling. Yet, further research is needed to investigate the possible contribution of hemodynamic or metabolic effects of sGC stimulators on memory performance.

1. Introduction

Evidence is accumulating that second messenger molecule cyclic guanosine monophosphate (cGMP) is important in memory processes in general, and for long-term potentiation (LTP) in particular (for a detailed overview, see [1]). Hence, an approach to enhance memory processes is by increasing intracellular cGMP. Under physiological circumstances, nitric oxide (NO) stimulates cGMP production by binding to soluble guanylate cyclase (sGC), inducing a conformational change which allows sGC to catalyse the conversion of guanosine triphosphate (GTP) into cGMP [2–4].

Physiologically, sGC-cGMP signalling is not only important for memory functioning but is also a pivotal pathway for healthy vascular functioning, which includes endothelial cell functioning and vascular smooth muscle cell (VSMC) relaxation [5,6]. The vascular system and central nervous system (CNS) interact at the blood–brain barrier (BBB), and at this level, sGC is involved in the communication between neurons and blood vessels to ensure optimal vascular functioning in the brain [7,8]. Pathologically, vascular functioning and memory functioning also intertwine, specifically at the level of vascular cognitive impairment (VCI) [9,10]. Interestingly, many of the underlying pathological processes described to underly VCI, can be related back to sGC e.g. endothelial dysfunction and a leaky BBB [11–13]. Therefore, sGC may be a novel target for treating cognitive impairments in VCI.
sGC can be targeted with sGC stimulators to increase cGMP production. These stimulators mimic the action of NO and stimulate sGC both independently of and synergistically to NO [14,15]. Currently, there are two sGC stimulators clinically approved. Vericiguat is on the market for the treatment of heart failure with reduced ejection fraction [14], and is able to enhance memory despite its inability to cross the BBB [16]. Riociguat is approved for the treatment of pulmonary arterial hypertension [17,18], and was discovered in an attempt to optimize YC-1, the first sGC stimulator ever described [15]. YC-1 has been shown to significantly improve cognition in aged rats [19]. However, YC-1 also inhibits hypoxia-inducible factor 1α (HIF-1α), and many of the reported effects induced by sGC stimulation of YC-1 could also be the result of HIF-1α inhibition [20,21]. As a clinically improved, and more specific sGC stimulator than YC-1, riociguat could therefore hold better potential as a novel treatment for cognitive impairments.

To assess the cognition-enhancing potential of riociguat, the present study evaluated the effects sGC stimulator riociguat on the memory of both healthy mice and mice subjected to a pharmacological model for memory impairment using the muscarinic M1 receptor antagonist biperiden, in the object location task (OLT) and the Y-maze alternation task. Additionally, we investigated the potential of combination treatment with suboptimal doses of riociguat and donepezil, an acetylcholinesterase inhibitor (AChEI), thereby targeting differential neurochemical cascades. Donepezil is currently the most widely used drug on the market to reduce cognitive decline in AD patients [22]. We also measured the contribution of peripheral vascular effects by testing riociguat in a memory impairment model in the OLT using the cerebral vasodilator-stimulated phosphoprotein (VASP).

Taken together, the behavioural and biochemical measurements will provide more insight into the effects of acute sGC stimulation on memory performance and further elucidate the role of the sGC-cGMP-PKG signalling pathway in memory processes.

2. Methods

2.1. Animals

All procedures involving animals were carried out under the Dutch Experiments on Animals Act (EAA, amended 1996), in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and the European Directive (2010/63/EU) for animal experiments. The experimental protocol was approved after evaluation by the animal ethical committee of Maastricht University (licensed animal ethical committee: Ministry of VWS, GZBIVVB891845). The official protocol number for these studies was DEC 2013-059. All studies involving animals are reported in accordance with ARRIVE guidelines [23].

In total 40 male C57BL/6 mice were supplied by Charles River (Sulzfeld, Germany) and tested between 4 and 5 months of age. The average body weight at the beginning of the study was 28.0 g. An additional cohort of 40 mice of the same age was used for the combination study, with an average body weight of 28.8 g at the start of the study. A cohort of 24 additional mice 4 months of age was used for the sumatriptan studies, with an average body weight of 24.5 g at the start of the study. A total of 36, 4-months-old male mice (average weight of 23.2 g) were used for the biochemical studies. The study opted for male mice only, since previous research with object location testing has shown that the oestrous cycle in rodents is a confounding factor for a stable memory performance [24]. All animals were housed individually in standard green line Tecniplast IVC cages on sawdust bedding. The animals were housed on a reversed 12/12-h light/dark cycle (lights on from 19:00 h to 07:00 h) and received food and water ad libitum. The mice were housed and tested in the same room. A radio, playing softly, provided background noise in the room. Testing was performed between 09:00 h and 18:00 h, with a small desk lamp providing dim light in the room. Animals were allowed to acclimatize to the room and individual housing for at least a week before testing commenced. The experimenter was always blind to the conditions that were being tested. For detailed sample size calculations, please see Supplemental Methods section 2.1.

Both research staff and animal facility caretakers monitored the animals daily with a full check-up at least once a week for signs of distress. Monitoring parameters included weight, facial expression, faeces, activity, and fur condition. If the animal showed signs of distress the veterinarian would be contacted and humane endpoints would be considered if distress was severe or long-lasting according to pre-defined scoring parameters determined in collaboration with the designated veterinarian. One animal within the sumatriptan cohort died prior to the start of experiments due to a spontaneous seizure.

2.2. Treatments

For details on the behavioral testing compounds, see the Supplemental Methods section 2.2. Both riociguat and biperiden were dissolved in 0.5 % methylcellulose (98 % of the total end volume) with 2 % tween-80. Doses of 0.01, 0.03, 0.1 and 0.3 mg/kg of riociguat were administrated orally (p.o.) 30 min in advance, for both the OLT and the Y-maze alternation task. Biperiden was given i.p. 30 min before the task at a dose of 3 mg/kg which is the optimal dose for cognitive impairment in the OLT as found in a pilot study previously performed in our lab (data not shown). Donepezil was given p.o. 30 min before the learning trial in the OLT at 0.03, 0.1 and 0.3 mg/kg to find the sub-optimal dose. The combination treatment groups all received two p.o. injections 30 min before testing, with vehicle, riociguat or donepezil. Sumatriptan was dissolved in saline and administered at a dose of 10 mg/kg s.c. The animals in the sumatriptan cohort all received a s.c. and p.o. injection 30 min before testing with vehicle, riociguat, or sumatriptan. All solutions were prepared freshly at the day of the experiment and administered at 5 ml/kg.

2.3. Behavioural tasks: object location testing and the Y-maze

The OLT has been derived from, and is very similar to, the object recognition task (ORT) [25]. The OLT has been performed as previously described elsewhere [26]. For details on the apparatus and detailed procedures, see Supplemental Methods section 2.3. The Y-maze continuous alternation test is a single trial task that measures spatial working memory and was performed as described previously [27,28]. For details on the For details on the apparatus and detailed procedures, see Supplemental Methods section 2.4.

2.4. Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [29]. Statistical significance was set at P < 0.05 for all analyses. The readout parameters of the OLT are the times that mice spent exploring each object during T1 and T2 [30,31] The time (in seconds) spent in exploring the familiar and the moved object in T2 are represented as 'a′ and 'b′, respectively. Total exploration times for both objects were calculated during T1 (represented as 'e1′) and T2 (represented as 'e2′). Memory performance was calculated and represented as the discrimination index d2 (′−b/a′)/'e2′, a relative index which shows no correlation with e1 or e2, and ranges from −1 to 1 [30]. A minimum amount of exploration is required to show reliable memory performance [30]. Therefore, animals were removed from the analysis if they spent <6 s exploring the objects during T1 or T2. One sample t-tests were used to compare the d2 index of the conditions to zero (i.e. chance level) [31]. To evaluate group differences, a one-way ANOVA was performed between the d2 values of the conditions. In case of a significant ANOVA effect, post-hoc Dunnett’s t-tests were performed to compare the experimental conditions to the vehicle (24 h OLT) or
biperiden/sumatriptan + vehicle condition (1 h OLT).

To measure spatial memory, the percentage of alternations was calculated by dividing the number of triads made by the maximum possible alternations (i.e. total entries minus 2) multiplied by 100. A score of 50 % alternations is considered chance level. Therefore, a significantly higher percentage than 50 % is indicative of functional working memory. A minimal of 10 arm entries per mouse was considered sufficient to provide a reliable score. Therefore, mice with <10 arm entries were excluded from the analysis. One-sample t-tests were used to compare each condition to a score of 50 %. A one-way ANOVA was performed to evaluate differences between the conditions. When the overall ANOVA was significant, post-hoc Dunnett’s t-test were performed to compare the conditions to the biperiden + vehicle treatment.

2.5. Determination of drug plasma and brain concentrations

After behavioural analysis in the OLT and Y-maze 16 mice were used for pharmacokinetic measurements of both riociguat and its main circulating active metabolite M–1 (BAY 60–4552), of which the pharmacological activity is 1/3 to 1/10 of that of riociguat [17]. The analysis was performed at Agilux laboratories, Inc. (Worcester, MA, USA), and detailed procedures can be found in Supplemental Methods section 2.5.

2.6. Surface protein biotinylation and sample preparation

For the biochemical study a total of 36 mice were used, who were habituated to the OLT according to the procedure described above. To evaluate brain plasticity markers, mice first received acute treatment with the behaviourally active dose of riociguat or vehicle. 30 min after injection the mice underwent the first trial of the OLT (T1) as a learning paradigm and subsequently were sacrificed either 1.5 h or 24 h after T1, i.e. 2 h or 24 h after the injection. Animals were sacrificed by means of cervical dislocation, the brains were excised and both hippocampi were isolated as described previously to prepare for biotinylation and processing [32]. For detailed descriptions on the biotinylation and isolation process, see Supplemental Methods section 2.6.

2.7. Western blotting

Surface protein fractions and their corresponding total protein samples (8 µg) were resolved in 10 % SDS-PAGE and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Veenendaal, The Netherlands). The membranes were blocked (50 % Odyssey blocking buffer in PBS, Li-Cor, Lincoln, NE, USA) for 1 h at room temperature, followed by overnight incubation with the primary antibodies at 4 °C. Membranes were subsequently incubated by secondary antibodies for 1 h at room temperature: goat anti-rabbit IRDye 800 and donkey anti-mouse IRDye 680. Membranes were visualized using the Odyssey CLx Infrared Imaging System (Li-Cor, Lincoln, NE, US) and protein bands were quantified using ImageJ (https://imagej.nih.gov/ij/). Raw intensity measures were normalized to GAPDH or β-actin to control for loading differences. Outliers were excluded based on a Dixon Q-test for outliers. The group means of riociguat treatment and vehicle treatment were compared at each timepoint by a t-test for equal means.

3. Results

3.1. Riociguat enhances long-term memory

No differences were found in exploration times during the learning or test trial (see Supplemental Table S2). If given vehicle treatment, mice were not able to remember the object locations after 24 h as is indicated with a d2 that is not different from chance level (i.e. zero, one-sample t-test). When administered a dose of 0.03 mg/kg riociguat, memory performance was significantly better than chance, indicating well-functioning spatial memory. None of the other tested doses were able to improve memory performance above chance (Fig. 1). Additionally, a one-way ANOVA showed a treatment effect on memory performance (F4,68 = 4.19; P < 0.01). Post-hoc Dunnett’s t-tests showed that mice treated with 0.03 mg/kg riociguat performed better compared to vehicle indicating better long-term memory performance.

3.2. Riociguat attenuates a biperiden-induced short-term memory deficit

No differences were found in exploration times during the learning or test trial (see Supplemental Table S2). After a 1 h interval, short-term memory performance of vehicle + vehicle treated mice was significantly higher both compared to chance (one-sample t-test) and 3 mg/kg biperiden (one-way ANOVA (F5,76 = 5.213; P < 0.001) with Dunnett’s t-tests) which indicates a biperiden-induced memory impairment. Mice treated with 0.03 mg/kg riociguat or 0.1 mg/kg riociguat were able to overcome this biperiden-induced memory impairment both compared to chance and 3 mg/kg biperiden (one-way ANOVA with Dunnett’s t-tests). This indicates that 0.03 and 0.1 mg/kg riociguat attenuated the biperiden-induced memory deficit (Fig. 2A).

3.3. Riociguat attenuates a biperiden-induced working memory deficit

The Y-maze continuous alternation task was performed to evaluate the acute effects of riociguat on spatial working memory after a biperiden-induced memory deficit (Fig. 2B). The mean alternation percentages were compared to 50 % alternations (chance level) by one sample t-tests. Vehicle + vehicle treated mice performed significantly better than both chance (one-sample t-test) and 3 mg/kg biperiden, which indicates a successful biperiden-induced working memory impairment (one-way ANOVA (F5,48 = 4.441; P < 0.01) with Dunnett’s t-tests), 0.01 mg/kg and 0.03 mg/kg riociguat treatment attenuated the biperiden-induced memory deficit, showing higher performances than chance (one-sample t-test) and 3 mg/kg biperiden (post-hoc Dunnett’s t-tests). 0.1 mg/kg riociguat treatment had an intermediate effect on

![Fig. 1. Riociguat enhances long-term memory in a 24 h interval OLT. Hashes indicate a difference from zero (one-sample t-test): ### P < 0.001. A difference from the vehicle condition is indicated with asterisks (one-way ANOVA with post-hoc Dunnett’s t-test): ** P < 0.01. Riociguat was injected p.o. 30 min before T1. Data are represented as mean + SEM with dots representing the individual datapoints.](image-url)
working memory performance: animals performed better than chance but not better than biperiden-treated animals. Lastly, 0.3 mg/kg riociguat treatment did not attenuate biperiden-induced impairment, as the animals did not perform higher than chance level or better than animals treated with 3 mg/kg biperiden only.

3.4. Riociguat interacts with sumatriptan to reverse sumatriptan-induced memory impairments

In this experiment, the effects of riociguat on memory impairment induced by the cerebral vasoconstrictor sumatriptan were measured (for a dose–response of sumatriptan, see Supplemental Fig. S1). No differences in exploration times were found during T1 and T2 (see Supplemental Table S3).

For short-term memory, sumatriptan treatment impaired memory performance compared to vehicle + vehicle (independent samples t-test) and indicated by similar performance to chance. 0.03, 0.1 and 0.3 mg/kg riociguat treated animals attenuated sumatriptan-induced short-term memory impairments, performing higher than chance (one-sample t-test) and sumatriptan only (one-way ANOVA (F4,70 = 6.425; P < 0.001) with Dunnett’s t-tests). This attenuating effect could not be found for 0.01 mg/kg riociguat + sumatriptan (Fig. 2A).

For long-term memory, vehicle + vehicle and vehicle + sumatriptan treated animals’ memory performance was similar to chance (one-sample t-test). 0.03 mg/kg riociguat enhanced memory performance while 0.1 mg/kg riociguat treatment did not increase memory performance compared to chance. However, when combined with sumatriptan, 0.1 mg/kg enhanced memory performance, while 0.03 mg/kg riociguat treatment did not. A two-way ANOVA modelling the effect of riociguat treatment, sumatriptan impairment and the treatment * impairment interaction, found a significant effect of treatment (F2,98 = 4.284; P < 0.05) and treatment*impairment (F2,98 = 6.213; P < 0.01), while no effects of impairment by itself could be found (F1,98 = 0.393; n.s.). Dunnett’s t-tests showed that 0.03 mg/kg riociguat + vehicle treated animals showed better memory performance compared to vehicle + vehicle treated animals. Furthermore, 0.1 mg/kg riociguat + sumatriptan treatment resulted in better memory performance compared to vehicle + vehicle treated animals (Fig. 3B).

3.5. Combined sub-optimal doses of riociguat and donepezil enhance long-term memory

To evaluate the effects of combined drug treatment of riociguat with donepezil, a 24 h OLT was performed with the sub-optimal doses of these drugs given in combination. Based on Fig. 1, 0.01 mg/kg riociguat was found to be the sub-optimal dose. Exploration times did not differ during T1 or T2 (see Supplemental Table S4). Only the combination of riociguat and donepezil enhanced long-term memory, as seen compared to chance (one-sample t-test) and compared to vehicle (one-way ANOVA (F2,34 = 3.116; P < 0.05) with Dunnett’s t-test). Treatment with sub-optimal doses of riociguat and donepezil alone did not enhance memory (Fig. 4).

3.6. Plasma and brain concentration of riociguat

Total plasma (Cp) and total brain (Cb) concentration, and the brain-to-blood ratios (Cb/Cp) for both riociguat and its metabolite M–1 are listed in Table 1. The behaviourally active dose of 0.03 mg/kg riociguat
Discrimination index (d2)

| Condition               | Discrimination Index |
|-------------------------|----------------------|
| Vehicle + Vehicle       | -0.5                 |
| Sumatriptan + Vehicle   | 0.0                  |
| Sumatriptan + 0.01 mg/kg Riociguat | 0.5               |
| Sumatriptan + 0.03 mg/kg Riociguat | 1.0               |

Administered at a dose of 10 mg/kg. Both sumatriptan (s.c.) and riociguat (p.o.) were injected 30 min before T1. Data are represented as mean ± SEM with dots representing the individual datapoints. A) The discrimination index (d2) is a measure for short-term memory performance in the 1 h interval OLT. Hashes indicate a difference from zero (one sample t-test): ** P < 0.01; *** P < 0.001. A difference from the sumatriptan + vehicle condition is indicated with asterisks (one-way ANOVA with post-hoc Dunnett’s t-test): * P < 0.05; ** P < 0.01; ### P < 0.001. B) The discrimination index (d2) is a measure for long-term memory performance in the 24 h interval OLT. Hashes indicate a difference from zero (one sample t-test): ** P < 0.01; ### P < 0.001. A difference from the vehicle + vehicle condition is indicated with asterisks (two-way ANOVA with post-hoc Dunnett’s t-test): * P < 0.05; ** P < 0.01.

Fig. 3. Riociguat interacts with sumatriptan-induced memory impairments in (A) the 1 h interval OLT and (B) the 24 h interval OLT. Sumatriptan was always administered at a dose of 10 mg/kg. Both sumatriptan (s.c.) and riociguat (p.o.) were injected 30 min before T1. Data are represented as mean ± SEM with dots representing the individual datapoints. A) The discrimination index (d2) is a measure for short-term memory performance in the 1 h interval OLT. Hashes indicate a difference from zero (one sample t-test): ** P < 0.01; ### P < 0.001. A difference from the sumatriptan + vehicle condition is indicated with asterisks (one-way ANOVA with post-hoc Dunnett’s t-test): * P < 0.05; ** P < 0.01; ### P < 0.001. B) The discrimination index (d2) is a measure for long-term memory performance in the 24 h interval OLT. Hashes indicate a difference from zero (one sample t-test): ** P < 0.01; ### P < 0.001. A difference from the vehicle + vehicle condition is indicated with asterisks (two-way ANOVA with post-hoc Dunnett’s t-test): * P < 0.05; ** P < 0.01.

Discrimination index (d2) appears detectable in the brain only 0.5 h after administration. However, the ratio between plasma and brain concentration of riociguat is equally low for every condition, indicating no or poor brain penetration of riociguat.

3.7. Effect of riociguat on plasticity markers and VASP

Treatment with riociguat did not affect the dynamics of GluA1-containing AMPA receptors, and no difference was observed for PSD95, synaptophysin, BDNF, or CREB phosphorylation both 2 h and 24 h after the treatment (Supplemental Fig. S2 and S3). In contrast, riociguat did increase VASP activation 2 h after treatment, an effect that could no longer be detected 24 h after riociguat treatment (independent samples t-test, Fig. 5).

4. Discussion

4.1. Riociguat enhances spatial memory in healthy and biperiden-induced memory impaired mice

0.03 mg/kg riociguat treatment counteracted natural forgetting, and biperiden-induced short-term memory and working memory impairments. 0.1 mg/kg riociguat was also fully effective in biperiden-induced short-term memory impaired animals. All other doses were either ineffective or showed an intermediate effect. This indicates that riociguat has a very narrow efficacy dose-range window for improving memory in healthy mice.

A combination of sub-optimal riociguat and donepezil dosing also effectively enhanced long-term spatial memory in the 24 h OLT. By combining two drugs that target different facets of memory-related neurotransmission, effects could be additive or synergistically effective [33]. A treatment strategy with sub-optimal doses might be preferred for the potentially lower side-effects, due to lower dosing. Further investigations are needed to substantiate this claim.

4.2. Riociguat reverses sumatriptan-induced memory impairments

Similar to biperiden-induced memory impaired mice, 0.03 mg/kg and 0.1 mg/kg riociguat enhanced short-term memory after sumatriptan-induced short-term memory impairments caused by cerebral vasoconstriction. Sumatriptan-induced short-term memory impairments were also previously shown in literature [34]. In contrast to biperiden, 0.3 mg/kg riociguat was also able to reverse sumatriptan-induced memory impairments. This indicates that the therapeutic window of riociguat widens under cerebral vasoconstriction. Perhaps, in vascularity healthy mice, overstimulation of the sGC-cGMP pathway is quickly reached and diminishes the possible positive effect of the drug, while under vascular impaired conditions, overstimulation may only occur at a higher dose due to a potential vascular mechanism of riociguat.

When measuring long-term memory, cerebral vasoconstriction induced by sumatriptan shifted the dose–response curve of riociguat to the right, i.e. a higher dose of 0.1 mg/kg was needed to enhance long-term memory in the 24 h interval OLT compared to the dose of 0.03 mg/kg riociguat required for long-term memory enhancement in healthy mice in. A two-way ANOVA further revealed a significant interaction effect between sumatriptan and riociguat, indicating that the working mechanisms of sumatriptan and riociguat interact, which further indicates a vascular mechanism for the memory enhancing effects of riociguat.

As a consequence of the very specific range of effectiveness after riociguat administration under vascularity healthy conditions, the level of sGC stimulation needed to obtain optimal memory enhancing effects might be missed easily in clinical conditions where the human dose is not corrected for body mass. This might why riociguat was unable to enhance memory in young adults either given alone or after biperiden treatment at translationally relatively high doses [35]. Perhaps riociguat would be more effective in subjects with reduced cerebral vascular health.
Table 1.

| Interval | 0.5 h | 1.5 h |
|----------|-------|-------|
| **liciguat (0.03 mg/kg)** | | |
| C_p (ng/ml) | 2.87 (0.29) | 1.32 (0.79) |
| C_o (ng/g) | 0.15 (0.15) | BQL |
| C_C_p | 0.07 (0.07) | n.a. |

| **liciguat (0.3 mg/kg)** | | |
| C_p (ng/ml) | 30.03 (3.04) | 15.00 (1.85) |
| C_o (ng/g) | 0.84 (0.10) | 0.64 (0.07) |
| C_C_p | 0.03 (0.00) | 0.04 (0.00) |

| **M–1 (0.03 mg/kg)** | | |
| C_p (ng/ml) | N.M. | N.M. |
| C_o (ng/g) | N.M. | N.M. |
| C_C_p | N.M. | n.a. |

| **M–1 (0.3 mg/kg)** | | |
| C_p (ng/ml) | 1.79 (0.27) | 1.91 (0.24) |
| C_o (ng/g) | 0.14 (0.14) | BQL |
| C_C_p | 0.14 (0.14) | n.a. |

Mean (+SEM) plasma and brain concentrations of liciguat and M–1 (0.3 and 0.03 mg/kg, p.o.) 0.5 and 1.5 h after administration. The lower limit of quantification for plasma and brain was 0.25 ng/ml and 0.4 ng/g, respectively. n.a.: not applicable; N.M.: not measured; BQL: below quantification limit.

4.3. Riociguat or its metabolite M–1 do not enter the brain

0.03 mg/kg riociguat was present at an average plasma concentration of 2.87 ng/ml 30 min after oral administration, and this was roughly reduced by half (1.32 ng/ml) 1.5 h after administration. The 10-fold higher dosage of 0.3 mg/kg riociguat showed a 10-fold increase in plasma concentration, also reduced by half 1.5 h after treatment. Interestingly, the brain concentration of riociguat was very low at a dose of 0.03 mg/kg 30 min after administration, and did not follow this 10-fold increase factor after a 10-fold increase of the dose. Additionally, the brain to blood ratio was close to 0.04 which is the ratio of the approximate cerebral blood volume relative to total unperfused brain volume [36]. This indicates that the riociguat measured in the brain samples was originating from the arteries within the brain tissue. We can conclude from these data that riociguat has very poor or no brain penetration.

To investigate whether riociguat’s activity is due to its main metabolite M–1 being active in the brain, the concentrations of M–1 were also evaluated. Interestingly, after administration of the high dose of 0.3 mg/kg, M–1 showed a sufficient brain/plasma ratio of 0.14 at 30 min after riociguat treatment. However, the plasma concentration of M–1 does not substantially change at 1.5 h after treatment, while the brain concentration becomes undetectable. This indicates that M–1 also has poor brain penetration. Taken together, the pharmacokinetic data question whether the memory enhancing effects of riociguat were directly mediated by a central effect.

4.4. No indication for a neuronal component to the memory enhancing mechanisms of riociguat

To investigate if a neuronal component underlies the effects of riociguat on memory performance, sGC-cGMP related neuronal markers were analysed in hippocampal tissue. AMPA receptor dynamics, CREB activation also has been shown to increase blood flow and might thus have an indirect effect on hippocampal function [40] and memory acquisition processes [42].

Analysis of pVASP and VASP in the hippocampus showed that riociguat treatment was able to increase the pVASP/VASP ratio indicative of pVASP activation at 2 h after treatment. Phosphorylation of VASP at Ser239 is a measure for PKG activity [38] and VASP has been connected to plasticity changes because of its effect on neuronal synapses, AMPA receptors and LTP [39–41]. However, similar plasticity changes were not observed in the current study. Therefore, it is possible that the VASP increase is measured in arteries innervating the hippocampus. VASP activation also has been shown to increase blood flow and might thus have an indirect effect on hippocampal function [40] and memory acquisition processes [42].

Schermuly et al. [43] showed that riociguat (10 mg/kg p.o.) did not affect mean arterial pressure, while decreasing right ventricular pressure and pulmonary resistance in two models for pulmonary arterial hypertension in rodents. Given that riociguat is an approved treatment for pulmonary hypertension, this indicates that the dose-dependent vascular effects of riociguat are not uniformly systemic. Therefore, at lower doses such as the memory enhancing doses of 0.03 mg/kg and 0.1 mg/kg riociguat, the effects of riociguat may very well occur at a cerebral microvascular level.

Of note, the current study used male mice only, since the study...
design was explorative in nature using riociguat as a potential cognitive enhancer for the first time. However, it is known that estrogen can influence both NO-sGC-cGMP signalling and memory performance in rodents [44-47]. Indeed, using only male mice is therefore a limitation to this study and future research with riociguat should include female mice, now that the efficacy of the sGC stimulator has been established.

5. Conclusions

In conclusion, sGC stimulation through riociguat treatment was able to enhance spatial memory in multiple healthy and pharmacologically impaired memory models despite the inability for riociguat to enter the brain. Interestingly, riociguat was found to interact with the cerebral vasoconstrictor sumatriptan by acting more strongly under cerebral vasoconstriction. Therefore, the memory enhancing effects of riociguat are likely derived from a cerebral (micro)vascular mechanism.

Ethics approval

All procedures involving animals were carried out under the Dutch Experiments on Animals Act (EAA, amended 1996), in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and the European Directive (2010/63/EU) for animal experiments. The experimental protocol was approved after evaluation by the animal ethical committee of Maastricht University (licensed animal ethical committee: Ministry of VWS, GZBIVVB981845). The official protocol numbers for these studies were DEC 2013-059 and 2018-006.

Author contributions

EN, BTJ: writing of manuscript, execution of experiments, data analysis, experimental design. EKA, NPG: execution of experiments, design of experiments, editing of manuscript. PRAH, DP, DAJM: support with execution of experiments, editing of manuscript. JGR, AB, HHHWS: experimental design, editing of manuscript. JP: experimental design lead, writing and editing of manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neulet.2022.136840.
