Blocking ROCK2 to improve recovery from brain injury (2019/2020)

Hypothesis and Aims:

***Hypothesis**: We hypothesised that blocking ROCK2 with the small molecule inhibitor KD025 will improve cognitive outcomes in a pre-clinical model of traumatic brain injury (TBI) by limiting peripheral inflammation and immune cell recruitment to the brain.*

The overall aims of these experiments were to determine whether ROCK2 blockade after brain injury:

1. ***Confers a therapeutic benefit, improving cognitive function after TBI,***
2. ***Confers neuroprotection, reducing damage to the cortical lesion site and areas undergoing degeneration after TBI (i.e. the hippocampus),***
3. ***Influences leukocyte infiltration into the damaged brain (i.e. hippocampus),***
4. ***Impacts on hippocampal neurogenesis.***

Methodology

Three-month-old wild-type mice underwent either sham surgery (craniotomy only) or a unilateral controlled cortical injury achieved by computer-controlled release of a stainless-steel piston impact on the exposed brain of anaesthetised mice using a TBI-0310 Impactor (Precision Systems and Instrumentation; 3.5m/s, 1mm depth, 400ms dwell time). After surgery, mice were oral gavaged once daily with vehicle (0.4% methylcellulose) or 200mg/kg KD025. These mice were also injected with BrdU (16.4mg/kg/mouse/day) for the first 3 days post-surgery for later measurement of in vivo neuron proliferation and survival.

To assess spatial learning and memory, mice were tested in the challenging active place avoidance (APA) task. The APA task assesses hippocampal-dependent spatial learning and memory and involves the animal continuously integrating visual cues to orientate itself in order to actively avoid a stationary shock zone (Vukovic *et al.* 2013). After completion of behavioural testing, mice were euthanised by trans-cardial perfusion fixation with brains kept in skull for high resolution magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) using a state-of-the-art 16.4 MRI Bruker Ultrashield Plus 700 WB Avance NMR spectrometer. After MRI/DTI imaging, brains were processed for detailed post-mortem histopathological analysis. This histopathological analysis included using immunohistological staining with antibodies specific for immature neurons (doublecortin; DCX), the proliferation marker BrdU, and neutrophils (Ly6B.2). Cell counts were performed using stereological methods with Stereo Investigator software.

Results

Blocking ROCK2 attenuates spatial learning and memory deficits induced by traumatic brain injury.

We first examined whether blocking ROCK2 via KD025 treatment can attenuate cognitive deficits induced by TBI. We utilized the APA task to test spatial learning in both sham and TBI treated with either vehicle or KD025 over a 5-day testing paradigm (**Figure 1A**). Sham operated vehicle mice quickly learnt to avoid the shock zone, as evident by their reduction in shock zone entries over 5 days, and improved performance (change in shock zone entries on day 5 versus day 1; **Figure 1B,C**). In contrast, vehicle TBI mice were not able to learn this task, making significantly more mistakes, entering the shock zone more often than their sham-operated counterparts (**Figure 1B,C**). Treatment of TBI mice with KD025 significantly improved APA task performance, with KD025-treated mice entering the shock zone significantly less over time and showed improvement in performance over time compared with vehicle-treated mice after TBI (**Figure 1B,C**).

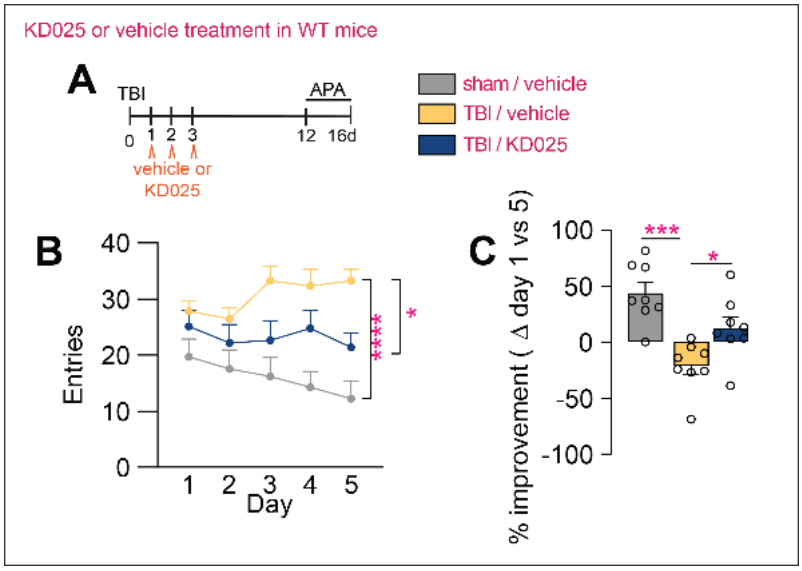

Figure 1. KD025 treatment attenuates spatial learning and memory deficits induced by traumatic brain injury.

*(**A**) Overview of experimental timeline. KD025 treatment or vehicle (0.4% methylcellulose) was given once daily for the first 3 days after surgery and mice subsequently tested in the APA task 12-16 days post-surgery.*

*(**B**) Entries into the shock zone across 5 days of APA testing. Vehicle-treated TBI mice fail to acquire this task [F(2,21), P<0.0001], but KD025 treatment improves cognitive abilities [Testing Day 5: 'TBI vehicle' vs. 'TBI KD025', P=0.013].*

*(**C**) APA performance plotted as 'percentage change' in the number of shock zone entries on Testing Day 5 versus Day 1 for individual mice. Note again the impact of TBI [F(2,21)=12.84, P=0.0002], and KD025 treatment improving APA performance compared with vehicle-treated TBI mice [P=0.047].*

*Data are represented as mean ± SEM. Statistics: repeated measures two-way ANOVA (B), one-way AVNOA (C) followed by Bonferroni post-comparison for all. n=8/group. *P<0.05, ***P<0.001, ****P<0.0001.*

Blocking ROCK2 is neuroprotective and reduces damage to the hippocampus after traumatic brain injury.

Having established that blocking ROCK2 via KD025 treatment improves hippocampal-dependent spatial learning and memory outcomes after TBI, we next investigated KD025 treatment effects on brain damage to the cortical lesion site and underlying hippocampus (**Figure 2A**). Ex vivo MRI volumetric analysis revealed significant loss of cortical tissue which was not influenced by KD025 treatment (**Figure 2B**). TBI also resulted in significant loss of hippocampal tissue compared with sham-operated controls (**Figure 2C**). KD025 treatment significantly reduced hippocampal volume loss after TBI (**Figure 2C**). Hippocampal volume was found to be correlated with APA performance (**Figure 2D; Figure 1C**).

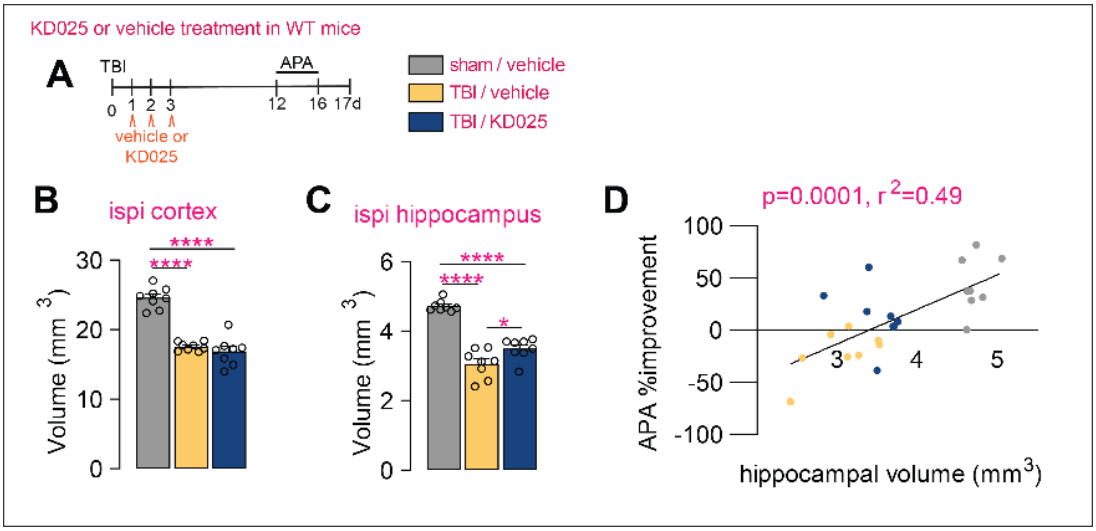

Figure 2. KD025 treatment reduces damage to the hippocampus after traumatic brain injury.

*(**A**) Overview of experimental timeline. KD025 treatment or vehicle (0.4% methylcellulose) was given once daily for the first 3 days after surgery and mice subsequently tested in the APA task 12-16 days post-surgery.*

*(**B**) T1/T2 ex vivo MRI measurements of the ipsilateral cortex for sham and TBI mice; asterisks indicate post-hoc results [F(2,21)=63.28, P<0.0001].*

*(**C**) T1/T2 ex vivo MRI measurements of the ipsilateral dorsal hippocampus for sham and TBI with vehicle or KD025 treatment; asterisks indicate post-hoc results [F(2,21)=62.93, P<0.0001; vehicle-treated TBI vs. KD025-treated TBI, P=0.031].*
*(**D**) Pearson's correlation between ipsilateral hippocampal volumes and percentage improvement in APA task performance (P=0.0001, r_2=0.49).*

*Individual mice are indicated as dots. Gray dots = sham-operated vehicle-treated mice; yellow coloured dots = vehicle-treated TBI mice, blue dots = KD025-treated TBI mice (**D**). Data are represented as mean ± SEM. Statistics: one-way AVNOA followed by Bonferroni post-comparison for all (**B,C**), Pearson's correlation (**D**). n=8/group. *P<0.05, ***P<0.001, ****P<0.0001.*

Blocking ROCK2 attenuates white matter damage induced by traumatic brain injury.

Having established that KD025 treatment attenuates secondary hippocampal degeneration, we next investigated whether therapeutic effects extended to attenuating damage to white matter tracks after TBI. We performed ex vivo diffusion tensor imaging and demonstrated that the hippocampal commissure and corpus callosum were negatively affected after TBI, with significantly reduced fractional anisotropy (FA) values compared with sham-operated controls (**Figure 3A,B**). KD025 treatment significantly attenuated FA value reductions for the hippocampal commissure compared with vehicle-treated TBI mice (**Figure 3B**). FA measures for the hippocampal commissure and corpus callosum were found to be correlated with percentage improvement in APA task performance (**Figure 3C-D**). No changes in any DTI measures were found for the anterior commissure, thus serving as an internal control (**Figure 3B,F,J**). DTI measures for the anterior were not correlated with APA performance (**Figure 3E,I,M**). When investigating the pathological drives underlying the FA changes, we found reductions in axial diffusivity (AD) and increases in radial diffusivity (RD) for the hippocampal commissure and corpus callosum after TBI (**Figure 3F,J**). KD025 treatment significantly attenuated changes in both AD and RD measures for the hippocampal commissure (**Figure 3F,J**). AD measures for the hippocampal commissure and corpus callosum were correlated with APA performance (**Figure 3G,H**). RD measures for the corpus callosum, but not the hippocampal commissure, were correlated with APA performance (**Figure 3K,L**)

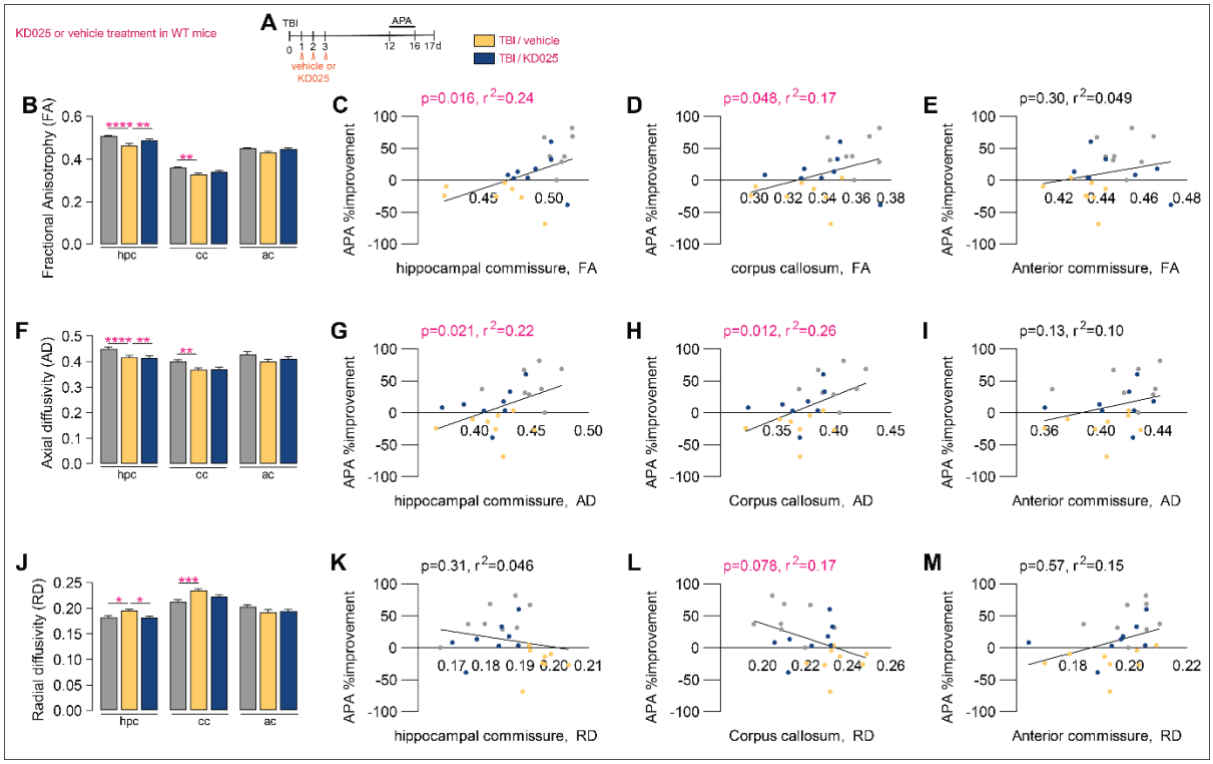

Figure 3. KD025 treatment attenuates white matter damage induced by traumatic brain injury.

*(**A**) Overview of experimental timeline. KD025 treatment or vehicle (0.4% methylcellulose) was given once daily for the first 3 days after surgery and mice subsequently tested in the APA task 12-16 days post-surgery.*

*(**B**) Fractional anisotropy (FA) values for the hippocampal commissure (hpc), corpus callosum (cc) and anterior commissure (ac) 17 days after TBI [$F(2,63)=21.73$, $P<0.0001$].*

*(**C-E**) FA values for the hippocampal commissure (**C**) and corpus callosum (**D**), but not the anterior commissure (**E**), were correlated with APA task performance.*

*(**F**) Axial diffusivity (AD, $\lambda_{||}$, $10^3 mm^2 s^{-1}$) for the hippocampal commissure, corpus callosum and anterior commissure [$F(2, 63) = 11.95$, $P<0.0001$].*

(G-I) AD values for the hippocampal commissure (G) and corpus callosum (H), but not the anterior commissure (I), were correlated with APA performance.

*(**J**) Radial diffusivity (RD, $\lambda_{\perp}$, $10^3 mm^2 s^{-1}$) values for hippocampal commissure, corpus callosum, and anterior commissure [$F(2, 63) = 5.10$, $P=0.0088$].*

*(**K-M**) RD values for the corpus callosum (**K**), but not the hippocampal commissure (**L**) or anterior commissure (**M**), were negatively correlated with APA performance.*

*Data are represented as mean ± SEM. Statistics: two-way ANOVA followed by Bonferroni post-comparison for all (**B, F, J**), Pearson's correlation (**C-E, G-I, K-M**); asterisks indicate post-hoc comparison. Gray dots = sham-operated vehicle-treated mice; yellow coloured dots = vehicle-treated TBI mice, blue dots = KD025-treated TBI mice. n=8/group. *P<0.05, ***P<0.001, ****P<0.0001.*

Blocking ROCK2 reduces neutrophil infiltrate into the hippocampus and supports hippocampal neurogenesis traumatic brain injury.

Having established that KD025 treatment significantly attenuates hippocampal damage following TBI, we next examined whether KD025 treatment effects the extent of neutrophil infiltration in the injured hippocampus following TBI (**Figure 4A**). When staining for Ly6B.2 (7/4 antigen), a *N*-glycosylated cell surface protein that is primarily expressed by peripheral neutrophils, we show significant recruitment of Ly6B.2$_{pos}$ infiltrate into the hippocampus after TBI compared with sham-operated vehicle-treated mice (**Figure 4B**). KD025-treated mice significantly reduced neutrophil presence in the ipsilateral hippocampus after TBI (**Figure 4B**).

Given our previous work demonstrating an important role for immature (4-week-old) granule cells in hippocampal-dependent spatial learning under TBI conditions (Willis et al., 2020), we next examined whether KD025 treatment effects the numbers of newly generated immature neurons expressing the microtubule-associated protein doublecortin (DCX) (Rao et al., 2004). We found that, irrespective of treatment, TBI mice had significantly less immature DCX$_{pos}$ neurons compared to sham-operated controls (**Figure 4C**). Notably, however, KD025-treated mice had significantly more DCX$_{pos}$ neurons compared with vehicle-treated mice after TBI (**Figure 4C**). The number of DCX$_{pos}$ immature neurons was significantly and positively correlated with APA performance (**Figure 4D**) Pulse-chase experiments with the mitogen BrdU revealed that KD025 treatment supported the survival of newly generated immatures neurons that were born post-injury (i.e. DCX$_{pos}$/BrdU$_{pos}$ neurons; **Figure 4E**).

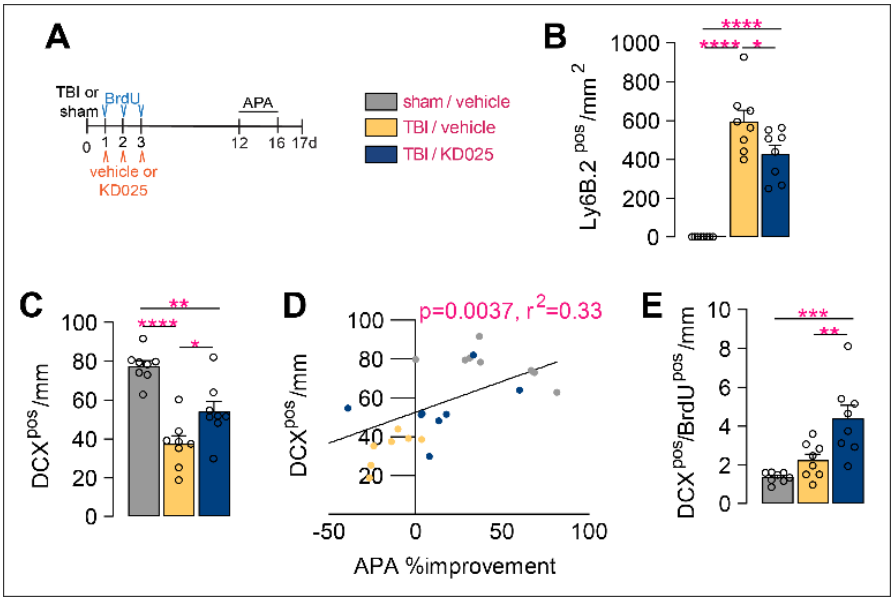

Figure 4. KD025 treatment reduces neutrophil infiltrate into the hippocampus and supports hippocampal neurogenesis traumatic brain injury.

*(**A**) Overview of experimental timeline. KD025 treatment or vehicle (0.4% methylcellulose) was given once daily for the first 3 days after surgery. BrdU was given twice daily for the first 3 days post-surgery and mice collected a day after the completion of APA testing at 17 days post-surgery.*

*(**B**) Quantification of Ly6B.2$_{pos}$ cells in the ipsilateral hippocampus after surgery [F(2,21)=51.94, P<0.0001].*

*(**C**) Quantification of DCX$_{pos}$ immature neurons in the ipsilateral granule cell layer after surgery [F(2,21)=22.17, P<0.0001].*

*(**D**) DCX$_{pos}$ immature neurons numbers are correlated with performance in the APA task after surgery.*

*(**E**) Quantification of immature neurons born post-surgery (i.e. DCX$_{pos}$/BrdU$_{pos}$) at 17 days post-surgery [F(2,21)=12.62, P=0.0003].*

*Data are represented as mean ± SEM. Individual mice are indicated as dots. (**D**) Gray dots = sham-operated vehicle-treated mice; yellow coloured dots = vehicle-treated TBI mice, blue dots = KD025-treated TBI mice. Statistics: one-way AVNOA followed by Bonferroni post-comparison (**B,C, E**), Pearson's correlation (**D**). n=8/group. *P<0.05, **P<0.01; ***P<0.001, ****P<0.0001.*