NEIL1 and NEIL2 DNA glycosylases modulate anxiety and learning in a cooperative manner in mice

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Oxidative DNA damage in the brain has been implicated in neurodegeneration and cognitive decline. DNA glycosylases initiate base excision repair (BER), the main pathway for oxidative DNA base lesion repair. NEIL1 and NEIL3 DNA glycosylases affect cognition in mice, while the role of NEIL2 remains unclear. Here, we investigate the impact of NEIL2 and its potential overlap with NEIL1 on behavior in knockout mouse models. Neil1−/− Neil2−/− mice display hyperactivity, reduced anxiety and improved learning. Hippocampal oxidative DNA base lesion levels are comparable between genotypes and no mutator phenotype is found. Thus, impaired canonical repair is not likely to explain the altered behavior. Electrophysiology suggests reduced axonal activation in the hippocampal CA1 region in Neil1−/− Neil2−/− mice and lack of NEIL1 and NEIL2 causes dysregulation of genes in CA1 relevant for synaptic function. We postulate a cooperative function of NEIL1 and NEIL2 in genome regulation, beyond canonical BER, modulating behavior in mice.
Cells in tissues and organs are continuously subjected to oxidative stress originating both from exogenous and endogenous sources such as reactive oxygen species (ROS), ionizing radiation, UV radiation, and chemicals, amongst others. The brain is especially susceptible to oxidative stress due to a high metabolic rate, low levels of antioxidant enzymes, and high levels of iron. Thus, repair of oxidative damage in the genome of postmitotic neurons is supposed to be critical for proper brain function. The hippocampus is a brain area critical for learning and memory formation and is also involved in anxiety. Increasing evidence shows that oxidative stress and defective DNA repair affects the hippocampus and leads to cognitive impairment. Oxidative stress has also been implicated in depression and anxiety. In mammalian cells, oxidative DNA damage is predominantly repaired via the base excision repair (BER) pathway. The enzymes in this pathway have been shown to be important for protection against neuronal cell death following induced ischemic brain damage. NEIL1 and NEIL2 are two of five DNA glycosylases specific for oxidative base lesions and the substrate specificities for these DNA glycosylases are partially overlapping. NEIL1 has broad substrate specificity and removes both pyrimidine- and purine-derived lesions such as 4,6-diamino-5-formamidopyrimidine (FapyA), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), guanidinohydantoin (Gh), spiroiminodihydantoin (Sp), and thymine glycol (Tg) from DNA. NEIL2 primarily removes oxidation products of cytosine such as 5-hydroxy-cytosine (5-ohC) and 5-hydroxy-uracil (5-ohU), also excised by NEIL1. NEIL1 and NEIL2 mRNA is homogeneously distributed and ubiquitously expressed in human and murine brain, indicating a role of NEIL1 and NEIL2 in DNA maintenance in most areas of the brain. NEIL1 and NEIL2 DNA glycosylases recognize and remove small base lesions (reviewed in). To date, eleven mammalian DNA glycosylases have been identified. NEIL1 and NEIL2 in DNA maintenance in most areas of the brain.
assay, there was a tendency to reduction in lesions/mutations in Neil1−/− Neil2−/− hippocampi, as compared to WT (P = 0.0856) (Supplementary Fig. 2). In sum, our data suggest that the behavioral phenotype observed in Neil1−/− Neil2−/− mice were not caused by impaired canonical BER.

Although the total steady-state levels of mutagenic oxidative DNA lesions were unaltered in hippocampi from adult mice lacking NEIL1 and/or NEIL2, mutations accumulated during development could still explain the phenotype at adulthood. To test this possibility, we applied whole-genome deep sequencing of hippocampal DNA from adult (6-month-old) male mice to determine mutation profiles. A DNA sequence variant analysis was performed using the WT hippocampus sample as the reference genome. We found a modest increase in DNA sequence variants genome-wide (Fig. 2d) that were evenly distributed across all chromosomes in all the three mutants (Fig. 2e). Variants were detected in all genomic regions with the majority occurring in non-coding regions, such as intergenic regions and
introns (Fig. 2f). Analysis of base-pair changes in SNPs showed a normal distribution with C/G to T/A transitions being the most frequent, most likely due to deamination of 5mC and C to thymine and uracil, respectively (Fig. 2g). These results indicate that lack of NEIL1 and/or NEIL2 does not lead to a genome-wide hypermutator phenotype in the hippocampus.

**Reduced axonal activation in stratum oriens of Neil1\(^{-/-}\)/Neil2\(^{-/-}\) hippocampus.** To assess potential changes in excitatory synaptic transmission and cell excitability that could possibly explain the altered behavior in NEIL1/NEIL2-deficient mice, we recorded in either stratum radiatum (SR) or stratum oriens (SO) and simultaneously in stratum pyramidale (SP) in the CA1 region of hippocampal slices from adult (4-month-old) male Neil1\(^{-/-}\)/Neil2\(^{-/-}\) and WT mice. We decided to focus on the hippocampal CA1 subfield due to its prominent role in both spatial information coding and anxiety regulation\(^{11,12,35,36}\). The stimulation intensities necessary to elicit prevelleys of given amplitudes (0.5, 1.0, and 1.5 mV) tended to be higher, though not statistically significant, in SR of Neil1\(^{-/-}\)/Neil2\(^{-/-}\) mice compared to WT mice (Fig. 3a). Similar tendencies were observed in SO, and for one of the prevelley amplitudes (0.5 mV), the difference between mutant and control mice reached statistical significance (Fig. 3f). Measuring the field excitatory postsynaptic potential (fEPSP) as a function of the same prevelley amplitudes showed that Neil1\(^{-/-}\)/Neil2\(^{-/-}\) animals evolved fEPSPs similar to those obtained in WT mice, in both SR (Fig. 3b, e) and SO (Fig. 3g, j). Furthermore, postsynaptic excitability, measured as fEPSPs necessary for generating a population spike, was not significantly changed in Neil1\(^{-/-}\)/Neil2\(^{-/-}\) mice compared to WT mice in SR (Fig. 3c, e) or SO (Fig. 3h, j). In the sum, the results do not support any major differences between the mutant and WT mice in excitatory synaptic transmission (Fig. 3b, g) or postsynaptic excitability (Fig. 3c, h) in either of the two strata examined. However, in SO, slightly altered axonal activation (Fig. 3f) could indicate a reduction in fiber density, number of afferent fibers or a differential receptor composition in receptor subunits in Neil1\(^{-/-}\)/Neil2\(^{-/-}\) mice compared to WT.

To further characterize excitatory synaptic transmission in the hippocampal CA1 region, we measured paired-pulse facilitation (PPF)\(^{37}\), a short-lasting form of synaptic plasticity primarily attributed to changes in presynaptic Ca\(^{2+}\) homeostasis\(^{38}\). A comparison of PPF did not reveal any differences between the two genotypes in SR (Fig. 3d) or in SO (Fig. 3i).

We next analyzed the long-term potentiation of synaptic transmission (LTP) at CA3 to CA1 synapses in WT and Neil1\(^{-/-}\)/Neil2\(^{-/-}\) mice in SR and SO. Tetanic stimulation of the afferent fibers in either of the pathways produced a lasting, homosynaptic potentiation of the fEPSP slope of similar magnitude in Neil1\(^{-/-}\)/Neil2\(^{-/-}\) and control mice, when measured 40 – 45 min after the tetanizations (Fig. 3k, l). In both SR and SO, LTP in Neil1\(^{-/-}\)/Neil2\(^{-/-}\) mice was similar in magnitude to the corresponding pathways in WT mice.

**NEIL1 and NEIL2 differentially affect gene expression in CA1 with a potential relevance for synaptic function, plasticity, and composition.** We recently reported hippocampal transcriptional changes in mice lacking OGG1 and MUTYH DNA glycosylases\(^{16}\). We therefore asked whether NEIL1 and NEIL2 DNA glycosylases could similarly act as transcriptional regulators within the hippocampus to modulate synaptic transmission and behavior. As for the electrophysiology, we focused on the CA1 subfield of the hippocampal formation due to its role in spatial learning and anxiety\(^{11,12,35,36}\). We applied whole-genome sequencing of RNA isolated from the pyramidal layer of CA1 of adult (3–6-month-old) male mice by laser capture microdissection (Fig. 4a), a method, which offers supreme tissue specificity\(^{39}\). A moderate number of differentially expressed genes (DEGs) were detected, the largest amount in Neil2\(^{-/-}\) and Neil1\(^{-/-}\)/Neil2\(^{-/-}\) mice (Fig. 4b, Supplementary Fig. 3d–f). Notably, there were no significant changes in the expression of other oxidative DNA glycosylases, such as Ogg1, Neil3 and Nth1 (Supplementary Fig. 3d–f), indicating that there is no compensatory upregulation of these repair genes in mice lacking NEIL1 and/or NEIL2. Similar numbers of up- and downregulated genes were found in all genotypes (Fig. 4c). While there was almost no overlap in DEGs between Neil1\(^{-/-}\)/Neil2\(^{-/-}\) and Neil2\(^{-/-}\)/ mice, we found most overlapping DEGs between single- and double-knockout mice (Fig. 4d). Exploratory data analysis identified two samples to be clear outliers (Supplementary Figs. 3g and 4). These two outliers (red arrows, Supplementary Fig. 3g) were excluded from the group comparison analysis. A reactome-pathway analysis showed the nuclear receptor signaling pathway (R-MMU-38328) to be significantly overrepresented in Neil1\(^{-/-}\)/Neil2\(^{-/-}\) CA1. Of note, all three isotypes of the orphan nuclear receptor Nr4a were downregulated in Neil1\(^{-/-}\)/Neil2\(^{-/-}\) mice, whereas the nuclear receptors Nr1d1 and Nr1d2 were upregulated (Fig. 4e). While four of these five nuclear receptors were similarly differentially regulated in Neil2\(^{-/-}\), none of them were altered in Neil1\(^{-/-}\)/CA1, pointing to a NEIL2-dependent regulation of nuclear receptors. The top10 downregulated genes in Neil1\(^{-/-}\)/Neil2\(^{-/-}\) mice largely overlapped with those of Neil1\(^{-/-}\) mice, whereas upregulated genes overlapped mainly with those of Neil2\(^{-/-}\) mice (Fig. 4f). Among the up- and downregulated DEGs we identified four genes as immediately relevant to synaptic function according to their QuickGO annotation\(^{40}\) (Fig. 4f). While three of them (Npbrw1, Htr3a and Fxyd2) play a role in a very specific subset of receptor systems and synaptic membrane elements, Npas4 is a well-characterized master regulator of inhibitory synapse development\(^{41}\). The latter was differentially regulated distinctly in Neil1\(^{-/-}\) and Neil1\(^{-/-}\)/Neil2\(^{-/-}\) mice.
animals, but not in Neil2−/− animals, indicating a mainly NEIL1-dependent regulation of this gene (Fig. 4f). Differences observed in group comparisons were also visible at a single animal level (Fig. 4g). To further thematically cluster the DEGs found in the different genotypes, we performed a gene ontology biological processes enrichment analysis (PANTHER release 2020-07-28, GO database release 2020-07-16, DEGs log2fold(abs) >0.3, p < 0.05). All NEIL-deficient mice showed an enrichment of several GO-terms immediately relevant to central nervous system function (colored GO-terms, Supplementary Fig. 3a–c), further highlighting the relevance of NEIL DNA glycosylases in CA1 transcription regulation. Interestingly, DEGs of Neil1−/−, Neil2−/− and Neil1−/−Neil2−/− mice showed enrichment for high-expressed genes (48%, 41% and 45%, respectively), as represented by the number of DEGs.
Fig. 2 Unchanged steady-state levels of oxidative DNA base lesions and no hypermutator phenotype in Neil1−/− Neil2−/− hippocampus.

a Hippocampus was isolated from WT and NEIL-deficient mice and DNA damage and mutation levels estimated by various methods. b HPLC-MS/MS analysis of 5-ohC in hippocampal, genomic DNA. Data are shown as mean ± SEM. (individual mice are represented by circles). n = 5 WT, 5 Neil1−/−, 10 Neil2−/− and 6 Neil1−/−Neil2−/− mice. c DNA damage levels in hippocampal tissue by alkaline comet assay analysis. Data are shown as mean of gel medians (circles indicate gel median for each mouse; 50 comets x 3 gels scored per mouse) ± SEM. n = 4 mice per genotype. *p = 0.0291 and 0.0495 for Neil2−/− and Neil1−/−Neil2−/− vs. WT, respectively, by two-way ANOVA/Sidak. d-g DNA samples from four mice of each genotype were pooled and subjected to whole-genome deep sequencing followed by mutation profile analysis (for details, see Methods). d DNA sequence variants, e Chromosomal distribution of DNA sequence variants, f Genomic region distribution of DNA sequence variants, and g Base changes count of SNPs in NEIL-deficient vs. WT hippocampus. SNP, single nucleotide polymorphism; Ins, insertions and Del, deletions.

Fig. 3 Reduced axonal activation in stratum oriens of Neil1−/− Neil2−/− hippocampus. a-j Synaptic transmission, excitability and paired-pulse facilitation (PPF) in stratum radiatum (SR; a-e) and stratum oriens (SO; f-j) of Neil1−/− Neil2−/− and WT mice. a, f Stimulation strengths necessary to elicit prevolleys of given amplitudes (0.5, 1.0, and 1.5 mV). b, g fEPSP amplitudes as a function of the same three prevolley amplitudes. c, h The fEPSP amplitudes necessary to elicit a just detectable population spike. d, i PPF ratio from the two genotypes at an interstimulus interval of 50 ms. e, j Recordings from stratum pyramidale (SP) elicited by paired-pulse stimulation (50 ms interstimulus interval). Arrowheads indicate the population spike thresholds in control (black) and Neil1−/−Neil2−/− (magenta) mice. Circles indicate prevolleys preceding fEPSPs in control (black) and Neil1−/−Neil2−/− (magenta) mice. Each trace is the mean of five consecutive synaptic responses elicited by different stimulation strengths. a-d, f-i Data are shown as mean ± SEM and n values represent total hippocampal slices per genotype (4-6 per mouse). Measures in individual slices are indicated with circles superimposed on the mean bars. a, b n = 20 WT for each of the stimulation strengths and 20, 20, 19 Neil1−/−Neil2−/− for 0.5, 1.0, and 1.5 mV, respectively. c n = 20 WT and 19 Neil1−/−Neil2−/−. d n = 20 for both genotypes. f, g n = 16, 16, 15 WT and 20, 20, 16 Neil1−/−Neil2−/− for 0.5, 1.0, and 1.5 mV, respectively. h n = 16 WT and 19 Neil1−/−Neil2−/−. i n = 16 WT and 20 Neil1−/−Neil2−/−. j P = 0.019 for prevolley of 0.5 mV, by linear mixed model analysis. k, l Normalized and pooled fEPSP slopes evoked in hippocampal slices from WT and Neil1−/−Neil2−/− mice in SR (k) and SO (l). The tetanized pathways are shown as circles and the untetanized control pathways are shown as triangles. Arrows indicate time points of tetanic stimulation. Data are shown as mean ± SEM and n-values represent total hippocampal slices per genotype (4-6 per mouse). k n = 10 WT and 11 Neil1−/−Neil2−/− l n = 9 WT and 8 Neil1−/−Neil2−/−.
in the 75% upper quartile of all sequenced genes. In contrast, less than 1.5% of DEGs in the mutant mice were low-expressed genes as demonstrated by the number of DEGs in the 25% lower quartile of all sequenced genes (Supplementary Fig. 5).

Altered synaptic composition in Neil2−/− and Neil1−/−Neil2−/− mice. Based on the transcriptome results showing differential regulation of factors relevant for synaptic composition, we decided to examine the excitatory and inhibitory transmitter systems within the CA1 subregion of the hippocampal formation by immunohistochemistry. We chose to study the NMDA- and GABA-receptors due to their reciprocal interaction with both Npas4 and Nr4a-isoforms and their previously shown association with NEIL-deficiency14. As a first approximation, we picked an immunohistochemistry-approach looking specifically into the CA1 subregion of the hippocampus of 3–6-month-old male mice (see also Methods and Discussion).

Within the tetrameric structure of the NMDA-receptor complex (Supplementary Fig. 6, illustration), regulatory subunits such as NR2A (GRIN2A) and NR2B (GRIN2B) determine the receptor’s electrophysiological properties and are seen as important mediators of synaptic plasticity42. We therefore primarily examined these two subunits of the NMDA receptor. Across SP, we found significantly reduced NR2A-reactivity in Neil2−/− and Neil1−/−Neil2−/− mice compared to WT (Supplementary Fig. 6a). NR2A reactivity was also significantly lower within SO in Neil1−/−Neil2−/− mice compared to WT (Supplementary Fig. 6a). As for the NR2B subunit, reduced reactivity was observed across SP of Neil2−/− mice only (Supplementary Fig. 6b). A low NR2A/NR2B-ratio has previously been reported to enhance both memory formation43 and LTP44. We observed a significantly reduced NR2A/NR2B ratio exclusively in Neil1−/−Neil2−/− mice, with the most prominent reduction across SO (SO, Δ2.558; SR, Δ1.685; SP, Δ1.053) (Supplementary Fig. 6c), the region that showed significantly reduced axonal activation (Fig. 3f).
Npas4 has been shown to coordinate inhibitory signaling via the GABA-A-receptor, both in vitro and in vivo. We chose to examine specifically the GABA-A-receptor alpha2 subunit (GABRA2) as it is involved in anxiety regulation via distinct intrahippocampal circuits. As for NR2A (Supplementary Fig. 6a), the GABRA2-reactivity was significantly reduced in Neil1−/−Neil2−/− mice compared to WT mice across SP (Supplementary Fig. 7a). In the Neil1−/− and Neil2−/− mice we observed a tendency to reduction in SP; however, this was not statistically significant (Supplementary Fig. 7a). While the expression of NR2A across SO and SR of Neil2−/− and Neil1−/−Neil2−/− mice showed a similar tendency to reduction as in SP (Supplementary Fig. 6a), the differences were less conclusive for GABRA2 in SO and SR (Supplementary Fig. 7a).

Next, we examined the expression of Postsynaptic density-95 (PSD-95), an abundant postsynaptic scaffolding protein associated with the NMDA-receptor complex. In line with reduced absolute levels of NR2A in SP of Neil2−/− and Neil1−/−Neil2−/− mice (Supplementary Fig. 6a) and NR2B in SP of Neil2−/− mice (Supplementary Fig. 6b), we found a tendency to reduced PSD-95 immunoreactivity in SP in both mutants (P = 0.0762 and 0.0983, respectively) (Supplementary Fig. 7b).

In sum, these results could point to a potential instability of NMDA-receptor architecture within the postsynaptic compartment in the context of NEIL1/NEIL2 deficiency (for limitations of this approach, see discussion).

Discussion

The current study revealed an altered behavioral phenotype in mice deficient in both the NEIL1 and NEIL2 DNA glycosylases, shown as increased locomotor activity in the OF test and the EZM, reduced anxiety in the EZM, and improved learning ability in the MWM test. We have previously reported similar observations in Ogg1−/−Mutyh−/− mice. However, in the Ogg1−/−Mutyh−/− mice, learning was impaired. Further, we recently demonstrated that mice carrying one deficient allele of Ogg1 exhibited poorer early-phase learning performance than WT mice using the Barnes maze, and that it was restored when the mice were subjected to oxidative stress by X-ray irradiation. Inactivation of NEIL3 DNA glycosylase induced an anxiolytic effect and a tendency to impaired learning in mice, however, without increased locomotor activity. In contrast, overexpression of the repair gene hMTH1, preventing 8-oxoG accumulation in the brain, also reduced anxiety in mice without an increase in activity level. Thus, it appears that DNA glycosylase affects processes involved in behavior and cognition in distinct ways. Canugovi and coworkers previously reported similar learning ability, but defects in short-term spatial memory retention in NEIL1-deficient mice. Correspondingly, no learning defects were observed in our NEIL1-deficient mice; however, memory was not affected either. A possible explanation to this discrepancy could be that the mice used in the present study were younger (6 months) than the mice tested by Canugovi and colleagues (9–33 months). Further, we have previously shown that Neil1 mRNA expression increases with age in mouse brains, suggesting that NEIL1 could be important for cognitive functions at a later stage than we have explored here. It may be argued that comparing mutant mice to control mice that are not littermates, could affect the outcome of the behavioral tests. However, since all lines were backcrossed onto the same background as control mice (C57BL/6 N), breeding of separate lines should not affect the results notably. The increased weight observed in Neil1−/− mice, but not in Neil2−/− and Neil1−/−Neil2−/− mice, could indicate that inactivation of Neil2 rescues the weight phenotype observed in the Neil1−/− mice, or it could be due to variable penetrence of the metabolic phenotype observed in the Neil1−/− mice. Since Neil1−/− and Neil2−/− mice show similar behavior, regardless of the weight differences, metabolic function and health status in Neil1−/− mice is unlikely to have an impact on behavior.

NEIL DNA glycosylases are assumed to be important for genome maintenance by preventing the accumulation of oxidative DNA damage. It is therefore reasonable to expect increased levels of oxidative base lesions and possibly mutations when these enzymes are lacking. In line with this, elevated levels of FapyA lesions, but not FapyG or 8-oxoG, were detected in brains from adult (9–22 months) NEIL1 KO mice. NEIL2 KO mice have also been shown to accumulate oxidized DNA bases in various organs, including the brain, but mainly in transcribed regions. In the present study, accumulation of hippocampal DNA damage was not detected in any of the DNA glycosylase-deficient strains studied and RNA sequencing analysis did not reveal any compensatory upregulation of other DNA glycosylases in CA1 of the hippocampus. Further, only a modest increase in DNA variants in NEIL-deficient hippocampi was found. Although a slightly higher number of variants were detected in the double KO compared to the single mutants, the number is too small (< 2500 per genome) for the double mutant to be characterized as a hypermutator. Similar observations were made in the spleen, liver, and kidney of NEIL1/NEIL2-deficient mice, which showed neither increased mutation frequencies nor cancer predisposition under normal physiology. Further, no global increase in 8-oxoG levels was detected in the hippocampus or hypothalamus of mice deficient in both the OGG1 and MUTYH DNA glycosylases.

Thus, impaired or reduced global (canonical) repair of oxidized DNA bases in brain regions involved in cognition is not likely to explain the altered behavioral phenotypes observed in DNA glycosylase-deficient mice. Intriguingly, Neil2−/− and Neil1−/−Neil2−/− mice showed reduced levels of DNA damage in the hippocampus. If NEIL2 plays a role in chromatin modulation, the NEIL2-deficient mice may contain more heterochromatin. Thus, we may speculate that reduced DNA damage is caused by a putative role of NEIL2 in processes making the chromatin more accessible to strand breaks.

We recently reported that transcriptional changes in the hippocampus of mice lacking OGG1 and MUTYH DNA glycosylases could be an underlying cause of the altered behavioral phenotype observed. Further, in Ogg1−/− hippocampus, the expression of three of 35 genes investigated was correlated to spatial learning in the Barnes maze. Thus, to begin to elucidate the mechanisms behind the behavioral alterations observed in the present study, we looked for changes in the hippocampal CA1 transcriptome. NEIL DNA glycosylases have previously been suggested to be involved in gene regulation by repairing preferentially transcribed genes and quadruplex DNA in promoter regions. In support of this, our RNA sequencing followed by transcriptome analysis revealed that in particular, highly expressed genes show differentially expression upon loss of NEIL DNA glycosylases. Notably, DEGs within the CA1 pyramidal layer of NEIL1/NEIL2-deficient mice referred to genes highly relevant for behavior, synaptic composition and function. Loss of NEIL2 appears to specifically affect Nr4a orphan receptors, with all three isoforms downregulated in Neil1−/−Neil2−/− mice, and largely overlapping with Neil2−/− mice. Consequently, DEGs in Neil1−/−Neil2−/− were significantly enriched in the nuclear receptor signaling pathway. In CA1, the nuclear receptor signaling pathway is particularly important for regulating excitatory synapse composition, dopaminergic signaling and, in general, processes of memory formation. Nr4a1 (Nurr7), whose function is enhanced when it forms heterodimers with Nr4a2 (Nurr1), interacts reciprocally with (excitatory) NMDA-receptor signaling. It regulates spine density and excitatory synapse distribution, especially at distal
dendritic compartments. Further, reduced expression of Nr4a2 has previously been linked to a hyperactive behavior phenotype in mice. This indicates a mechanistically relevant impact of NEIL1 and NEIL2 on these receptors to modulate adaptive behavior. As another example of NEIL1 and NEIL2 interacting with gene expression relevant for synaptic composition and function, we observed Npas4 to be downregulated both in \(\text{Neil}1^{-/-}\) and \(\text{Neil}2^{-/-}\) animals. Npas4 is prominently involved in regulating the excitatory-inhibitory balance within neural circuits, with a particular relevance for GABAergic (inhibitory) signaling. In sum, this suggests that NEIL1 and NEIL2 glycosylases jointly affect the expression of genes relevant for synaptic composition and function, with NEIL2 being prominently involved in nuclear receptor signaling and NEIL1 mainly involved in Npas4 regulation.

With Npas4 being a regulator in excitatory-inhibitory balance and Nr4a receptors interacting directly with the NMDA-receptor, we further examined the expression of the NMDA-receptor in the context of NEIL-deficiency by immunohistochemistry. Here, we focused on the regulatory subunits NR2A and NR2B due to their eminent role in determining receptor electrophysiological properties as well as its relevance in behavior and pathophysiology. The results we present here point to a potentially reduced NR2A/NR2B ratio in NEIL1/NEIL2-deficient mice compared to WT, which constitutes a further refinement of previously reported altered NMDA-receptor composition. This may partly explain the behavioral phenotype of improved spatial learning in NEIL1/NEIL2-deficient mice, since recent findings suggest a low NR2A/NR2B ratio to be associated with improved memory acquisition performance and enhanced LTP. However, we need to point out that our approach bears certain limitations requiring further studies to confirm the observations we report here: While we chose a highly subregion-specific approach with a fluorescent immunosignal-based quantification selectively within the regions of interest (see Methods), we cannot present subregion-specific data at a protein level confirming these results. Thus, we chose to present the immunohistochemistry data as part of the Supplementary Material (Supplementary Fig. 6). Confirmation of results could be done e.g. with a mass spectrometry approach looking specifically into CA1-SP laser dissecates; however, this is beyond the scope of this study.

The coupling between LTP in CA1 and spatial reference memory in the MWM has been questioned in recent studies. Thus, the unaltered LTP in the \(\text{Neil}1^{-/-}\) mice does not necessarily contradict the improved learning. While we did not observe differences in LTP, \(\text{Neil}1^{-/-}\) mice displayed electrophysiological changes in form of decreased axonal activation in SO of the hippocampal CA1 subregion. However, these differences were observed for one specific pre-volley amplitude only. Nonetheless, we think that this may point to a reduced number of afferent fibers in SO. Interestingly, recent evidence suggests that the inhibition of heterogeneously tuned excitatory afferent input to CA1 is beneficial for spatial coding. One could therefore speculate that a decrease in afferent fiber density may cause reduced excitatory input to CA1 in the context of NEIL1/NEIL2 deficiency, sufficient for improved spatial coding, at least in the very general spatial learning context of a MWM. However, spatial information coding is distinctly a network task involving all hippocampal subfields as well as the entorhinal cortex. Our study only examines the, albeit very important, CA1 subfield in detail and the behavioral read-out used for this study do not permit conclusions about more refined elements of spatial coding such as pattern completion.

Throughout our study, we used robust and strict statistical approaches and we do think that our experiments were sufficiently powered to draw the conclusions we present in this manuscript (see considerations on a priori/post-hoc power analyses in Methods). Yet, our RNAseq approach relied on a relatively small sample size (\(n = 3\)) due to the technically challenging, time- and cost-intensive experimental approach. The statistical approach we used to analyze our RNAseq data takes this into account (DESeq2, see Methods), thereby allowing to draw conclusions with a reasonable reliability. We recommend nonetheless that future studies looking into the behavioral, electrophysiological and biomolecular description of new transgenic mouse models in DNA repair anticipate a high data variance, as observed e.g. in Fig. 1, and take this into account in a priori power calculations.

Recently, NEIL1 was identified as a potential reader of oxidized cytosine derivatives, and both NEIL1 and NEIL2 were suggested to potentially cause gene reactivation by an alternative BER pathway for DNA methylation. Furthermore, both proteins were shown to promote substrate turnover by TDG (Thymine-DNA glycosylase) during DNA demethylation. This suggests a role in gene regulation, possibly involving epigenetics. In light of this, the behavioral phenotype observed in NEIL1/NEIL2-deficient mice does not seem to be caused by impaired canonical repair of oxidative base lesions. Instead, our results point to a NEIL1/NEIL2-dependent regulation of synaptic factors both at RNA and protein level that is not explained by the enzymes' function in DNA repair, but rather their noncanonical contribution to gene regulation.

**Methods**

**Experimental model and subject details.** All experiments were approved by the Norwegian Animal Research Authority and conducted in accordance with the laws and regulations controlling experimental procedures in live animals in Norway and the European Union’s Directive 2010/63/EU. NEIL1 KO (\(\text{Neil}1^{-/-}\)), NEIL2 KO (\(\text{Neil}2^{-/-}\)) and NEIL1/NEIL2 DKO (\(\text{Neil}1^{-/-}\)\(\text{Neil}2^{-/-}\)) mouse models generated previously in our lab, were used throughout the study. Three mutant lines, all founder mice, were used for at least eight generations onto the C57BL/6 N background, were bred separately to obtain mice for experiments and C57BL/6 N mice were included as WT controls. Of note, breeding of separate lines reduces the number of mice needed for experiments and is in agreement with general practice, as long as the mouse are backcrossed onto the same background as control mice (https://www.jax.org/amx-mice-and-services/customer-support/technical-support/breeding-and-husbandry-support/considerations-for-choosing-controls). The mice were housed and bred in a 12-h-light/dark cycle at the Department of Comparative Medicine, Oslo University Hospital, Rikshospitalet, Norway, or the Comparative Medicine Core Facility, NTNU, Trondheim, Norway, with food and water ad libitum. The mice were housed with their littermates (max five in each cage). Throughout the study we have used male mice aged 3 to 6 months. This age group is usually referred to as mature adult and consists of mice that are post development, but not yet affected by senescence. It is a relatively homogeneous group when it comes to the parameters we have investigated in the present study. Specific age is stated in respective methods and results sections. Different cohorts of mice were used in each experiment, except for in behavioral studies where some of the mice were used in all three tests.

**Behavioral studies.** Behavioral studies were performed on 6-month-old mice. The mice were subjected to the behavioral tests in the following order: Open Field Maze (OFM), Elevated Zero Maze (EZM) and Morris Water Maze (MWM). The mice were used in all three tests, however, in three of the mice were included as tests are less time consuming than the MWM. The mice subjected to the MWM were weighed after the last probe test. The OF test monitoring general locomotor activity was conducted in an arena measuring 140 cm x 40 cm x 53 cm, where the middle of the arena, 20 cm x 20 cm, was defined as the center area zone. Mice were allowed to explore the arena for 5 min. The EZM task measuring activity and anxiety, was conducted on a circular runway 60 cm above the floor with four alternating open and closed areas. The mice were placed on the maze facing a closed area and allowed 5 min for exploration of the apparatus. An open area entry was defined as 85% of the mice being inside an open area. Learning and memory were monitored using the MWM. Testing was carried out in a white circular pool, 120 cm in diameter and filled 2/3 with white, opaque water (Sika/Lateq liquid, Sika, Norway) kept at 22 ± 1 °C. Using visual cues, the mice learned to find a hidden escape platform, 11 cm in diameter and located at a fixed position 0.5–1.0 cm below the water surface, during repeated daily sessions (days 1–4). The mice were released in the water facing the wall of the pool at four fixed positions in a pseudorandom sequence and given a maximum of 60 s to locate the hidden platform. Each mouse had eight trials each day in the training period,
four in the morning and four in the afternoon. After each block (four trials) the mouse was placed in a heated cage to dry before being returned to the home cage. On days when the test was not run, the mice were subjected to a tail-sacrifice (probe test) to test spatial memory. During retention trials, the escape platform was submerged to the bottom of the pool. A spatial bias for the target quadrant constitutes evidence for spatial memory. During all three behavioral tests, positions of the mice were tracked and stored by using ANY-maze video tracking system (Stoelting, IL, USA).

DNA damage analysis. HPLC-MS/MS analysis. DNA was isolated from hippocampi of 4-6-month-old WT and NEIL-deficient mice using DNeasy Blood and Tissue kit (Qiagen, cat. no. 80804), according to manufacturer’s protocol. Two µg of genomic DNA was enzymatically hydrolyzed to deoxyribonucleosides by incubation in a mixture of benzozene (Santa Cruz Biotechnology, sc-391121B), nuclease P1 from Penicillus citrinum (Sigma, N8630), and alkaline phosphatase from E. coli (Sigma-Aldrich, P9931) in 10 mM ammonium acetate, pH 6.0, 1.0 mM magnesium chloride buffer at 40 °C for 40 min. Three volume equivalents of ice-cold acetonitrile were added to the reactions after digestion was completed to precipitate proteinaceous contaminants. Following centrifugation at 16000 g at 4 °C for 40 min, the supernatants were collected in new tubes and dried under vacuum at room temperature. The resulting residues were dissolved in water for HPLC-MS/MS. Chromatographic separation was performed using a Shimazu Prominence LC-20AD HPLC system with an Ascentis Express C18 2.7 µm 150 × 2.1 mm i.d. column equipped with an Ascentis Express Cartridge Guard Column (Supelco Analytical, Bellefonte, PA, USA) with EXP Titan Hybrid Fertilize (Optimize Technologies Inc.). For analysis of unmodified nucleosides the following conditions were used: Flow was set to 0.4 ml/min; column temperature was 50 °C; type A elution was consisted of 5% B for 5 min, 5% B for 20 min, 25% B (0.1 % formic acid in methanol) at 0.16 ml/min, 40 °C. For analysis of 5-OH: 0.14 ml/min flowing start with 5% B for 0.5 min, followed with a gradient of 5–45% B for 7.5 min, finishing with re-equilibration with 5% B for 5.5 min. Online spectrometry detection was performed using an Applied Biosystems/MDS Sciex API5000 Triple quadrupole mass spectrometer (ABSciex, Toronto, Canada), operating in positive electrospray ionization mode. The deoxyribonucleosides were monitored by multiple reaction monitoring using the following mass transitions (m/z): 127.1 (dA), 152.1 (dG), 243.1 (dT), and 268.1 (dC) with 244.1 (dC) as a pseudo internal standard. Net Fpg-sensitive sites were calculated by subtracting the median comet tail score (50 comets × 3 replicate gels scored) was used to calculate the mean values per genotype. Flow cytometry and HPLC-MS/MS analysis were performed according to the GATK Best Practices recommendations using the Mutect2 variant caller, with KOs as case and WT for set to 10. Hard filtering was applied to get variant results of higher confidence. To identify strain-dependent genetic variation—i.e., variants inherited from the 129 strain and not completely lost through back-crossing with the C57BL/6 N strain—SNP and InDel data were loaded into the genome browser SegMonk (http://www.bioinformatics.babraham.ac.uk/projects/segmonk/) for further inspection. We defined 129-specific regions as having more than 50 detected SNPs or InDels per 600 KB bases and used this as a criterion in the “Read Count Quantitation using all Reads” probe extraction method in SegMonk. Individual regions satisfying this criterion were extracted and consecutive regions within the genome were joined to form the final 129-dependent regions. We confirmed enrichment of 129-dependent genetic variants within each region by identifying the SNPs that were present in dbSNP (build 137) and counting the number of times the SNP genotype matched the annotated 129 (129P/OlaHsd, 129Jvlv, or 129JvlvStrv strains) or black 6 (C57BL/6NJ) strain genotypes.

DNA mutation analysis Whole-genome deep sequencing. For each genotype, hippocampal genomic DNA from four 6-month-old male mice was isolated using DNeasy Blood and Tissue Kit (Qiagen, cat. no. 80804), pooled and sent to BGI Tech Solutions, Hong Kong, for whole-genome sequencing, including library construction and HiSeq4000 sequencing.

Identification of strain-dependent genetic variations. We identified SNPs and insertions/deletions (InDels) individually for mutant and WT samples. Specifically, the adapter sequence in the raw data was removed, and low-quality reads which had too many Ns (>10%) or low-quality score (≤5) was discarded. The remaining reads were aligned to the mouse reference sequence (mm10) using the Burrows-Wheeler Aligner (BWA)48. The alignment information was stored in RAM format files, which was further processed by fixing mate-pair information, adding read group information and marking duplicate reads caused by polymerase chain reaction artefacts. The variant calling steps included SNPs detected by SOAPsnp51 and small InDels detected by Sambtools/GATK49. In GATK, the caller UniﬁedGenotyper was used with the parameters stand_call_conf set to 50 and stand_emit_conf set to 10. Hard filtering was applied to get variant results of higher conﬁdence. To identify strain-dependent genetic variation—i.e., variants inherited from the 129 strain and not completely lost through back-crossing with the C57BL/6 N strain—SNP and InDel data were loaded into the genome browser SegMonk (http://www.bioinformatics.babraham.ac.uk/projects/segmonk/) for further inspection. We defined 129-specific regions as having more than 50 detected SNPs or InDels per 600 KB bases and used this as a criterion in the “Read Count Quantitation using all Reads” probe extraction method in SegMonk. Individual regions satisfying this criterion were extracted and consecutive regions within the genome were joined to form the final 129-dependent regions. We confirmed enrichment of 129-dependent genetic variants within each region by identifying the SNPs that were present in dbSNP (build 137) and counting the number of times the SNP genotype matched the annotated 129 (129P/OlaHsd, 129Jvlv, or 129JvlvStrv strains) or black 6 (C57BL/6NJ) strain genotypes.

Identification of mutations in NEIL-deficient hippocampi. Reads were filtered and aligned to the mouse genome as described above, and alignments were preprocessed according to GATK Best Practices recommendations using GATK version 3.5, including local realignment around InDels and recalibration of quality scores. For calling we used the MUTECT2 variant caller48, with KOs as case and WT as control. Mutations were considered significant if they were present in the test sample but absent in the control sample and where the difference is unlikely due to sequencing errors. We used MUTECT2 default parameters, which include rejecting candidates that in the control sample have (i) supporting reads numbering ≥ 2 or constituting ≥ 3% of the total reads (i.e., <34 total reads) and (ii) their quality score is ≥ 25. We used snpEff36 and Snpsift38 to annotate all SNPs and InDels found and discarded SNPs and InDels overlapping the 129-specific intervals for each sample.

Electrophysiology Slice/Sample preparation. Adult (4-month-old) WT and Neil+/−/Neil−/− mice were sacrificed with Suprane (Baxter) and the brains removed. Transverse slices (400 µm) were cut from the middle and dorsal portion of each hippocampus with a vibroslicer (Leica VT 1200) in artificial cerebrospinal fluid (ACSF, 4 °C, bubbled with 95% O2–5% CO2 containing (in µM): NaCl 124, KCl 1,25, KH2PO4 2, MgSO4 1, CaCl2, 26 NaHCO3 and 12 glucose. Slices were placed in an interface chamber exposed to humidity (pH 7.3) containing 2 mM CaCl2 for at least 1 h prior to the experiments. In some experiments, dl-2-amino-5-phosphopentanoic acid (AP5, 50uM; Sigma-Aldrich, Oslo, Norway) was added to the ACSF in order to block NMDA-receptor-mediated excitatory post-synaptic currents (<300 µA, 0.1 Hz) were delivered through tungsten electrodes, inserted into the middle and dorsal part of the hippocampal CA1 region. The presynaptic volley and the field excitatory post-synaptic potential (fEPSP) were recorded by a glass electrode (filled with ACSF)
placed in the corresponding synaptic layer (separated approximately 200 µm from the stimulation electrode) while another electrode placed in the pyramidal cell body layer (0.1–0.3 mm from the stimulation electrode) and a third electrode placed in the stratum radiatum of CA1. All electrodes were connected to the AAP-3000 amplifier (A-M Systems, WA, USA), and all experiments were done in parallel. For calibration of the EEG recorded from the stimulating electrode, a single 100 Hz tetanus were obtained (1 s, repeated four times at 5 min intervals). As standardization, the stimulation strength used for tetanus was just above the threshold for generation of a population spike in response to a single test shock. Synaptic efficacy was assessed by measuring the slope of the fEPSP in the middle third of its rising phase. Six consecutive responses (1 min) were averaged and normalized to the mean value recorded 1–4 min prior to tetanization. Data were pooled across animals of the same experimental group and pathway and are presented as mean ± SEM. All experiments were done in parallel in two separate electrophysiology setups. One setup was equipped with two Axoclamp2A amplifiers (low pass filtered at 3 kHz) (Molecular Devices, USA), custom-made 10X amplifiers (in house), Digitizer (National Instruments, USA) and custom-made programs for recording and analysis. The other setup was equipped with one Axoclamp2A amplifier (Molecular Devices, USA) and one Bio-logic VFI180 (Claix, France) (both low pass filtered at 3 kHz), custom-made 10X amplifiers (in house), Digitizer (National Instruments, USA), and custom-made programs for recording and analysis.

**Long-term potentiation (LTP) of synaptic transmission.** Orthodromic synaptic stimuli (50 µs, 300 µA) were delivered alternately through two tungsten electrodes (0.1-μM Ohm WPI, USA), one situated in the middle of SR and another in the middle of SO of the homocentrically orientated coronal slice. For the remaining 120 min, the RFP channel was observed for 40, 50, or 60 µs in order to define the stimulation/response range. A similar approach was used to elicit paired-pulse responses (50 ms interstimulus interval, the two stimuli being equal in strength). To assess synaptic transmission, we measured the amplitudes of the presynaptic volley and the fEPSP at the different stimulation strengths. During the analysis, we also used an additional stimulus to measure the presynaptic volley and the fEPSP at the different stimulation strengths. During the analysis, care was taken to use extrapolated measurements, which were within the apparent linear part of the I/O curves. Values from individual experiments outside the linear part of the I/O curves (prevoxy vs. fEPSP) were omitted when pooling the data. The population spike amplitude was measured as the distance between the maximal population spike amplitude and a line joining the maximum pre- and postspike fEPSP positivities. In order to pool data from the paired-pulse experiments, we selected responses to stimulation strength just below the threshold for eliciting a population spike on the second fEPSP.

**Antibody treatment.** Heat-mediated antibody retrieval was performed for 3 min at 99 °C in a 40 mM trisodium citrate (Merck, Darmstadt, Germany) solution, pH 6.0. Specimens were then left to cool down to room temperature inside this solution for another 27 min (30 min total exposure). 5% normal goat serum/bovine serum albumin (Bacterial Ohio, Norway) and subsequently an intraperitoneal, weight-adapted overdose of pentobarbital (200 mg/kg body weight). Intracardiac perfusion was performed with 0.9% saline (B.Braun, Melsungen, Germany) and 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were put into 4% paraformaldehyde/PBS solution for a minimum of 48 h at 4 °C for fixation. We sectioned brains at a thickness of 30 µm using a cryostat (Leica CM3050S, Nussloch, Germany) starting at a medio-lateral depth of 900 µm and continuing until the end of the tissue block. Slices were then stored at 4 °C in a PBS-solution containing 0.05% of Proclin (Merck, Darmstadt, Germany) until further processing.

**Confluent microscopy.** Imaging of stained slices was done using a Zeiss LSM880 confocal microscope. For confocal microscopy, a Plan-Apochromat 40x/1.4 Oil DIC M27 objective (Carl Zeiss, Jena, Germany) was used. An imaging square of 700 × 700 µm (x/y 2000 pixels of 0.35 µm each) and a z-interval of 0.5 µm was applied. A proximal and a distal imaging square was set within the CA1 region based on NeuN and DAPI stainings as an anatomical orientation (center of proximal square set at 0.25x total CA1 length measured from proximal end, center of distal square set at 0.75x CA1 length). Results displayed are averaged across proximal and distal squares. For each animal, one medial and one lateral brain slice was analyzed, amounting to a total of 4 imaging squares (2 proximal, 2 distal).

**RNAseq bioinformatic preprocessing.** Bioinformatic processing was performed using the BGII Genomics Co., Ltd. (Hong Kong, China) using the following workflow: a) filtering of low-quality reads using the software SOAPpK2; b) genome mapping with HISAT software; c) transcript reconstruction using StringTie and reference comparison with Cuffcompare, d) prediction of coding potential with CPC; e) SNP and INDEL detection with GATK; e) reference mapping with Bowtie2; f) calculation of gene expression levels using the RSEM software package; g) hierarchical clustering with hclust in R.
With respect to the nested data problem, all statistical analysis was done at an animal level (1 animal = 1 statistical unit). On a sideline, we observed a consistently different NeuN signal in NEIL2 deficient mice. Tissue quality, immunostaining protocol parameters, background signal and DAPI-counter-staining efficiency was identical in these samples compared to the other genotypes, so that a genotype-specific NeuN signal appears possible and, while beyond the scope of this manuscript, warrants further investigation.

Quantification of immunoreactivity. Imaris 9.3 (Bitplane, Zurich, Switzerland) was used to quantify immunoreactivity. We first created a 3D reconstruction of the whole z-plane dataset. The strata pyramidale/orient/riadium were identified as regions of interest using the “slice” tool in Imaris and copied to every z-plane accordingly. Based on the surface selection, a 3D-frame was created and the parameter of interest “masked” according to this frame. The Imaris “Spots Wizard” function was used to identify areas of synaptic reactivity within this masked channel (1 µm spot diameter, background subtraction applied according to local contrast). We then conducted a pilot-experiment for every immunohistochemical marker used, involving typically 4 different images. This was to make sure a biologically relevant signal is captured by the software. Based on this pilot experiment, a selection criterion based on the "quality" (see bitplane.com/imaris) filter in Imaris was defined and kept the same throughout the analysis. Imaris automated background subtraction was done for every specimen analyzed to account for intensity variations despite identical immunohistochemistry and confocal parameters. 2/3 of the region of interest had to be intact (i.e. not damaged by tissue cracks, covered by imaging artefacts etc.) in order to be included in the analysis. One exclusion was made based on this criterion (NR2A immunostaining, experiment, a selection criterion based on the biologically relevant signal is captured by the software. Based on this pilot experiment, a selection criterion based on the “quality” (see bitplane.com/imaris) filter in Imaris was defined and kept the same throughout the analysis. Imaris automated background subtraction was done for every specimen analyzed to account for intensity variations despite identical immunohistochemistry and confocal parameters. 2/3 of the region of interest had to be intact (i.e. not damaged by tissue cracks, covered by imaging artefacts etc.) in order to be included in the analysis. One exclusion was made based on this criterion (NR2A immunostaining, experiment, a selection criterion based on the biologically relevant signal is captured by the software. Based on this pilot experiment, a selection criterion based on the “quality” (see bitplane.com/imaris) filter in Imaris was defined and kept the same throughout the analysis. Imaris automated background subtraction was done for every specimen analyzed to account for intensity variations despite identical immunohistochemistry and confocal parameters. 2/3 of the region of interest had to be intact (i.e. not damaged by tissue cracks, covered by imaging artefacts etc.) in order to be included in the analysis.

The RNA sequence data generated and analyzed during the current study have been deposited in NCBI’s Gene Expression Omnibus (GEO), accession number GSE160621. The DNA sequence data have been deposited in European Nucleotide Archive (ENA), accession number PRR31108. The figure source data are available in Supplementary Data 1. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Magnar Bjørás (magnar.bjoras@ntnu.no).

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Author contributions

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Author contributions

M.B., K.S., J.Y., A.K., L.E., and G.S. directed the study and/or obtained financial support. G.A.H. and L.L. generated the NEIL1 KO mouse model in collaboration with the Norwegian Transgenic Center. R.S. generated the NEIL1 KO mouse model in collaboration with the Norwegian Transgenic Center. R.S. generated the NEIL2 KO mouse model and backcrossed and bred the mice. N.K. backcrossed and bred the mice in Trondheim. V.R., O.M. and M.D.S. designed behavioral experiments. V.M., R.S., S.V. and M.D.B. conducted behavioral experiments. A.D.R. analyzed behavioral results, including statistics. A.N.K. performed HPLC-MS/MS. A.K.O. and K.B.G. performed alkaline comet assay. K.S. performed PCR-based DNA damage assay. P.S. and K.S. analyzed DNA sequencing data. V.J. designed and conducted electrophysiological studies and analyzed the data obtained. N.K. designed the laser capture microdissection experiment. N.K., S.B.S. and M.S.F. performed the laser capture microdissection experiment. N.K. isolated RNA from laser-dissected samples. M.S.F. performed the immunostaining experiments. N.K. did the confocal imaging and subsequent Imaris-analysis as well as statistical analysis. W.W. performed qPCR. A.M.B. analyzed the RNAseq data acquired from BGI. A.M.B. and N.K. performed the gene ontology and pathway analysis of RNAseq data. N.K. did the single-gene QuickGo thematic analysis. N.K. and G.A.H. laid out, revised and structured all figures based on single plots and figures made by other authors. G.A.H., V.R., N.K., K.S. and M.B. wrote the manuscript with input from the other authors.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information

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