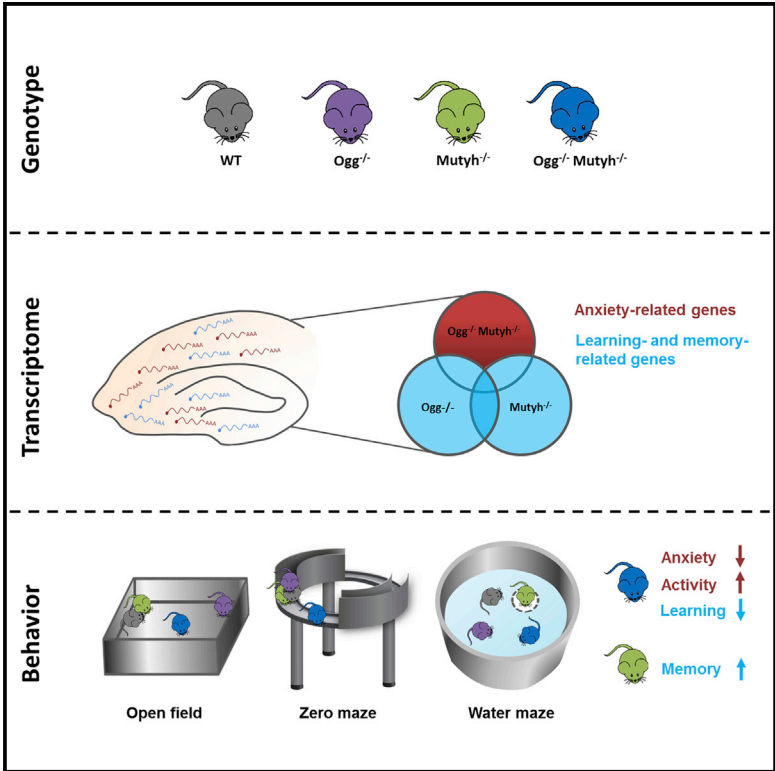# Cell Reports

Synergistic Actions of Ogg1 and Mutyh DNA Glycosylases Modulate Anxiety-like Behavior in Mice

Graphical Abstract

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In Brief

Ogg1 and Mutyh cooperate to prevent mutations caused by 8-oxoG. Bjørge et al. report increased activity, decreased anxiety, and impaired learning in $Ogg1^{-/-}Mutyh^{-/-}$ mice but unaltered 8-oxoG levels. Genes involved in anxiety and cognition are differentially expressed in $Ogg1^{-/-}Mutyh^{-/-}$ mice, suggesting Ogg1 and Mutyh modulate gene expression related to adaptive behavior.

Highlights

- $Ogg1^{-/-}Mutyh^{-/-}$ mice show increased activity, decreased anxiety, and impaired learning

- No apparent accumulation of 8-oxoG was found in mutant mouse brains compared to WT brains

- Differentially expressed genes in $Ogg1^{-/-}Mutyh^{-/-}$ brains are important for anxiety

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Synergistic Actions of Ogg1 and Mutyh DNA Glycosylases Modulate Anxiety-like Behavior in Mice

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SUMMARY

Ogg1 and Mutyh DNA glycosylases cooperate to prevent mutations caused by 8-oxoG, a major premutagenic DNA lesion associated with cognitive decline. We have examined behavior and cognitive function in mice deficient of these glycosylases. $Ogg1^{-/-}Mutyh^{-/-}$ mice were more active and less anxious, with impaired learning ability. In contrast, $Mutyh^{-/-}$ mice showed moderately improved memory. We observed no apparent change in genomic 8-oxoG levels, suggesting that Ogg1 and Mutyh play minor roles in global repair in adult brain. Notably, transcriptome analysis of hippocampus revealed that differentially expressed genes in the mutants belong to pathways known to be involved in anxiety and cognition. Esr1 targets were upregulated, suggesting a role of Ogg1 and Mutyh in repression of Esr1 signaling. Thus, beyond their involvement in DNA repair, Ogg1 and Mutyh regulate hippocampal gene expression related to cognition and behavior, suggesting a role for the glycosylases in regulating adaptive behavior.

INTRODUCTION

DNA is constantly threatened by reactive oxygen species (ROS), generated as byproducts of metabolic processes in the cell. Accumulation of oxidative DNA damage caused by ROS may lead to mutagenesis or cell death and is associated with aging, neurodegenerative disease, and cancer (Hegde et al., 2012; Canugovi et al., 2013). Base excision repair (BER) is the major pathway for removal of oxidative DNA base damage. BER is initiated by DNA glycosylases that recognize and excise damaged bases, leaving apurinic or apyrimidinic (AP) sites, which are subsequently removed by incision activities (i.e., 3′- and 5′-phosphoribodiesterases and AP lyases). Repair synthesis is completed by gap filling and ligation (Krokan and Bjørås, 2013).

One of the most premutagenic oxidative base lesions, 8-oxo-guanine (8-oxoG), is caused either by oxidation of G in DNA or by incorporation of 8-oxo-dGMP during replication. 8-oxoG occasionally pairs with A during replication, and this may lead to G:C to T:A transversion mutations. Two mammalian DNA glycosylases, Ogg1 and Mutyh, cooperate to prevent mutations caused by 8-oxoG. Ogg1 removes 8-oxoG when paired with C and Mutyh removes A mispaired with 8-oxoG (Michaels and Miller, 1992) (Figure S1). 8-oxoG has been shown to accumulate in aging animals, especially in organs with limited cell proliferation such as brain, kidney, liver, and lung (Møller et al., 2010), and elevated levels of 8-oxoG have been detected in brains from patients with Parkinson's disease, Alzheimer's disease, and Huntington's disease (Shimura-Miura et al., 1999; Wang et al., 2005; Polidori et al., 1999). Furthermore, mice deficient of Ogg1 and/or Mutyh show increased incidence of various cancers (Russo et al., 2004; Xie et al., 2004; Sakamoto et al., 2007; Tsuzuki et al., 2007). Thus, intact BER is of significant importance to prevent accumulation of 8-oxoG and its potentially fatal outcomes.

To begin to elucidate a possible role for 8-oxoG BER in regulating anxiety-like behavior and cognition, we analyzed Ogg1 and/or Mutyh DNA glycosylase-deficient mice by various behavioral tests including the open field and zero maze as well as the Morris water maze to examine hippocampal-dependent learning and memory. Transcriptome profiling by RNA sequencing was performed on naive mice in order to determine
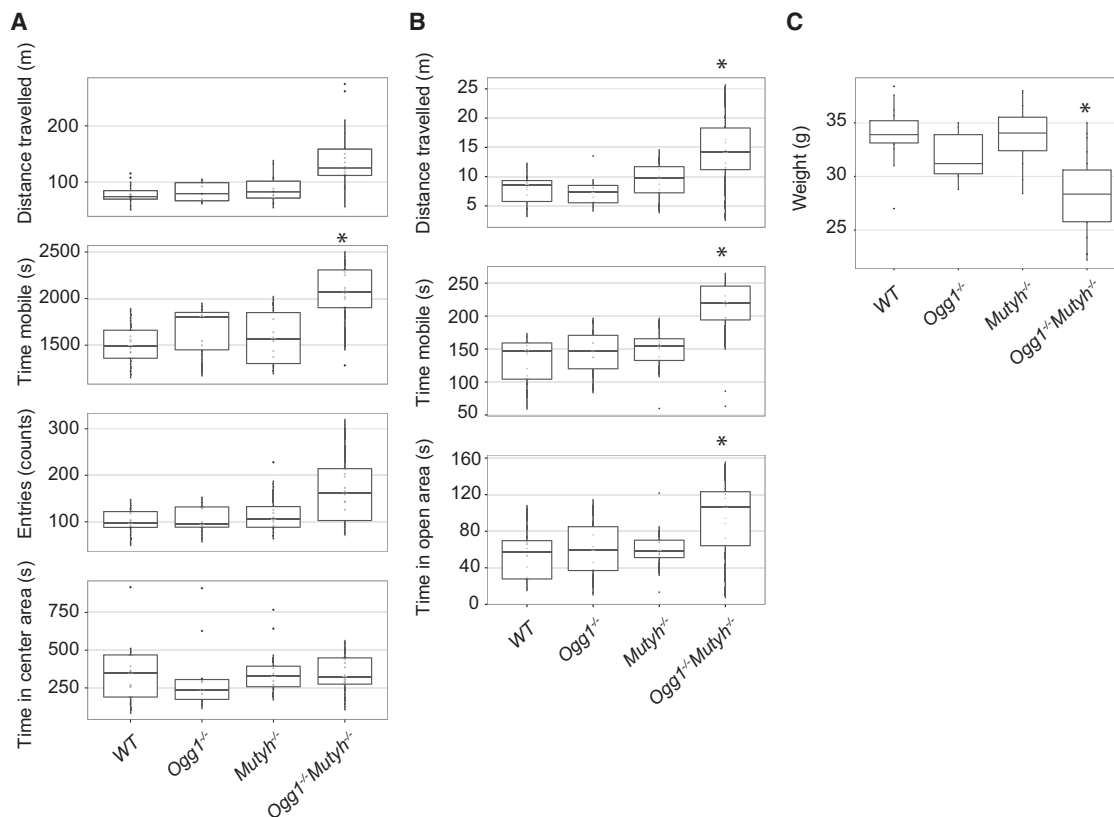
Figure 1. Increased Activity and Reduced Anxiety-like Behavior in Ogg1/Mutyh-Deficient Mice

(A) In the open field maze, the mice were allowed to explore freely for 45 min in an arena measuring 35 cm high × 40 cm long × 40 cm wide. An area of 20 cm long × 20 cm wide was defined as the center area zone. The $Ogg1^{-/-}Mutyh^{-/-}$ mice were significantly more mobile than the other genotypes (*p = 0.0002, 0.008, and 0.007 for $Ogg1^{-/-}Mutyh^{-/-}$ versus WT, $Ogg1^{-/-}$, and $Mutyh^{-/-}$, respectively; n = 11–22 mice per genotype).

(B) In the zero maze, the mice were allowed 5 min for exploration on a 5-cm-wide circular runway with alternating open and closed areas. The $Ogg1^{-/-}Mutyh^{-/-}$ mice were significantly more active and spent more time in the open areas than the other genotypes (*p = 0.0003, 0.0007, and 0.023 for distance traveled; *p = 0, 0.003, and 0.002 for time mobile; and *p = 0.003, 0.04, and 0.03 for time in open area for $Ogg1^{-/-}Mutyh^{-/-}$ versus WT, $Ogg1^{-/-}$, and $Mutyh^{-/-}$, respectively; n = 11–18 mice per genotype).

(C) Reduced body weight in $Ogg1^{-/-}Mutyh^{-/-}$ mice (*p = 0, 0.013, and 0 for $Ogg1^{-/-}Mutyh^{-/-}$ versus WT, $Ogg1^{-/-}$, and $Mutyh^{-/-}$, respectively; n = 11–18 mice per genotype).

(A–C) Data are shown in full, with overlaid boxplots representing the medians and interquartile ranges (IQR), and whiskers extending to a Tukey fence set at 1.5xIQR. Data were analyzed by post hoc family-wise multiple comparison of means (Tukey honest significant difference). See also Figure S2.

whether 8-oxoG DNA glycosylases influence the expression of genes related to adaptive behavior and cognitive function.

RESULTS

Increased Activity Level and Reduced Anxiety in Ogg1/Mutyh-Deficient Mice

General activity and movement were monitored in the open field test (Figure 1A). The $Ogg1^{-/-}Mutyh^{-/-}$ mice were more active than the wild-type (WT) and single knockout (KO) mice. They also showed a tendency to enter the center area zone more frequently than the other genotypes; however, they did not spend more time there. Increased activity was also observed in the zero maze (Figure 1B). In addition, the mice spent more time in the open-area zone, indicating less anxiety compared to the other genotypes. The $Ogg1^{-/-}Mutyh^{-/-}$ mice weighed in average less than the other mice (Figure 1C), and this may

have had an impact on the activity level of these mice. On the other hand, the reduced weight could be a consequence of the increased activity.

Altered Learning and Memory Performance in Ogg1/Mutyh-Deficient and Mutyh-Deficient Mice

Learning and memory performance was tested in the Morris water maze (Figure 2). The $Ogg1^{-/-}Mutyh^{-/-}$ mice needed significantly more time to learn the position of the escape platform as compared to WT mice (Figure 2A). However, memory did not seem to be affected, as the mean distance to the platform zone and the time spent in the target quadrant did not differ significantly from WT mice on days 5 and 12 (Figures 2B and 2C). In contrast, the $Mutyh^{-/-}$ mice learned the position of the platform at a rate similar to that of WT mice (Figure 2A), searched closer to the platform zone (Figure 2B), and spent significantly more time in the target quadrant than the other genotypes,
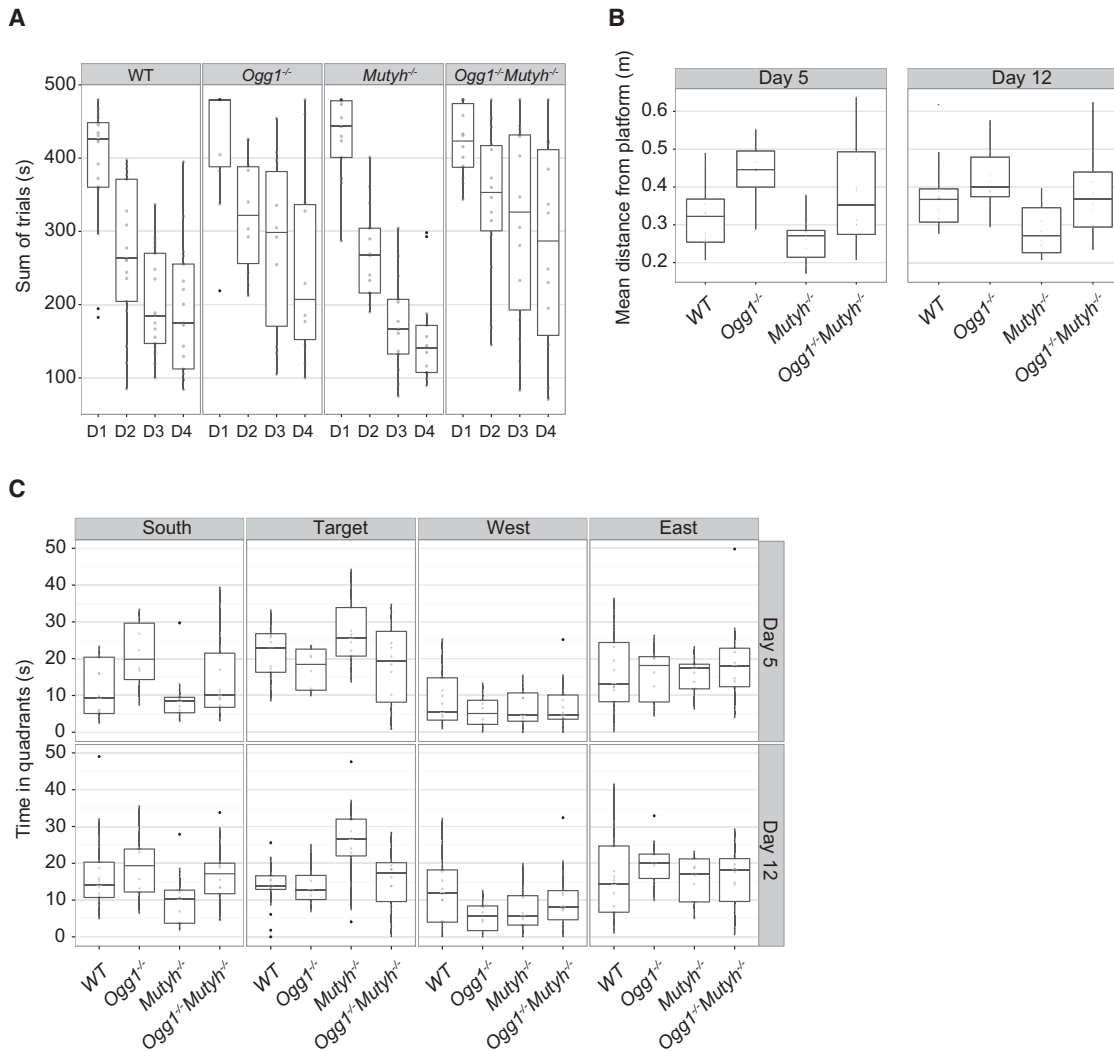
Figure 2. Altered Learning and Memory in Mice Deficient of Ogg1 and/or Mutyh

In the Morris water maze task, mice were trained to locate an escape platform hidden below the water surface (days 1–4) before memory was tested (days 5 and 12).

(A) $Ogg1^{-/-}Mutyh^{-/-}$ mice learned the position of the platform at a significantly slower rate than the $Mutyh^{-/-}$ (p < 0.010) and WT mice (p < 0.007; non-parametric pairwise Wilcoxon rank-sum test).

(B) The mean distance from the platform zone (Gallagher's measure) was significantly shorter for $Mutyh^{-/-}$ mice than for all other genotypes during probe trials on days 5 and 12 (p < 0.02; Tukey honest significant difference multiple comparison of means). No significant difference was seen between results on days 5 and 12.

(C) Time spent in the four quadrants of the tank during probe trials. The platform zone is located in the target quadrant. There is a significant difference between genotypes in terms of the proportion of swimming time spent in the target quadrant, with $Mutyh^{-/-}$ mice showing a stronger tendency to reside in the target quadrant on both days (p < 0.0011; for detailed statistics, see Supplemental Experimental Procedures).

(A–C) n = 10–17 mice per genotype. Data are shown in full, with overlaid boxplots representing the medians and interquartile ranges (IQR) and whiskers extending to a Tukey fence set at 1.5xIQR. See also Figure S2.

both on days 5 and 12 (Figure 2C). Taken together, these data suggest that the DNA glycosylases Ogg1 and Mutyh may adopt distinct roles in regulating behavior and cognitive function.

Brain Dimensions Were Not Affected in Mice Deficient of Ogg1 and/or Mutyh

Morphological examination of brain regions of importance for cognition could potentially elucidate the abnormal behavior observed in $Mutyh^{-/-}$ and $Ogg1^{-/-}Mutyh^{-/-}$ mice. Thus, brain

dimensions such as brain area, hippocampal area, and cortex thickness were measured by using MAP2 stained coronal brain sections (Figure S2D). No significant differences were found between WT and KO mice (Figures S2A–S2C).

No Accumulation of 8-oxoG in Hippocampus and Hypothalamus of Mice Deficient of Ogg1 and/or Mutyh

Accumulation of oxidative DNA damage has been reported both in aging animals and in animals lacking one or more DNA repair
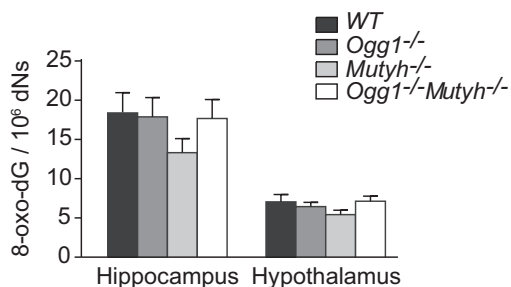
Figure 3. No Differences in Total Genomic 8-oxoG Levels in Mice Deficient of Ogg1 and/or Mutyh

Hippocampal and hypothalamic DNA from all four genotypes was subjected to LC-MS/MS analysis for quantification of 8-oxodG lesions. No significant differences were observed between the genotypes in the two brain regions. Data are presented as mean with SEM; n = 9–10 hippocampus and n = 4–5 hypothalamus.

enzymes (Møller et al., 2010). The hippocampus is a brain area critical for learning and memory and is also a key region for anxiety (Eichenbaum, 2013; Le-Niculescu et al., 2011). The hypothalamus plays an important role in stress response and anxiety as a member of the hypothalamus-pituitary-adrenal axis (Herman et al., 2003). To determine whether accumulation of 8-oxoG lesions could explain behavioral and cognitive differences in our mice, we measured 8-oxoG levels in the hippocampus and hypothalamus of WT and KO mice by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Surprisingly, we found no significant differences in global 8-oxoG levels between WT and KO mice in any of the brain regions examined (Figure 3), suggesting that other functions of the DNA glycosylases than repair of 8-oxoG are responsible for the observed effects on anxiety-like behavior and cognition.

RNA Sequencing Revealed Candidate Genes and Pathways Involved in Anxiety and Cognitive Functions in Mutant Mice

To elucidate the genome-wide transcriptional profiles, we applied RNA-sequencing analysis of the hippocampi from WT and mutant mice. Only differentially expressed genes (DEGs) with a minimum of 2-fold change in the expression level and p < 0.05 were selected. Overall, we identified 140 to 190 DEGs in the mutant mice as compared to WT mice, with the majority in each mutant (81%–97%) being upregulated genes (Figure 4A). Interestingly, a relatively large group of DEGs (63 genes) were common for all three mutants (Figure 4B). To identify DEGs that potentially contributed to the anxiolytic effect observed in $Ogg1^{-/-}Mutyh^{-/-}$ mice, we selected all genes that were differentially expressed in double KO (DKO) mice only. Of these, 43 were upregulated and 10 were downregulated (Figure 4C). Analysis using the PANTHER pathway classification system revealed that the greatest number of DEGs from this group of genes belongs to the gonadotropin-releasing hormone receptor pathway and the corticotropin releasing factor receptor signaling pathway, previously reported to be top candidate pathways involved in anxiety, specifically in the hippocampus (Le-Niculescu et al., 2011). In addition, we found several genes dysregulated in the DKO mice that in the same

study were listed among the highest-ranked candidates related to anxiety in the hippocampus (Figure 4D). Interestingly, the most highly upregulated gene in the DKO mice, Prl, has been shown to exert an anxiolytic effect when injected intracerebroventricularly in rats (Torner et al., 2001). Next, we selected all DEGs in the mutant mice for pathway analysis to identify pathways involved in cognitive functions. The majority of DEGs (58%) were not mapped to any pathways, but notably, 42% of the DEGs that showed pathway hits were enriched in pathways associated with learning and memory (Figure 4E). Among the top hits, we identified the Wnt- and the integrin-signaling pathways, which are essential for synaptic maintenance and neuronal function (Rosso and Inestrosa, 2013; Benson et al., 2000). In addition, the Alzheimer's disease/presenilin pathway was highly enriched suggesting that transcriptional changes in mutant mice parallel an Alzheimer's disease profile, a disorder characterized by deficits in learning and memory (Albert, 2002).

Ogg1 and Mutyh Repress Esr1 Signaling in Hippocampus

Very recently, Cho and colleagues demonstrated that transcriptional repression via inhibition of estrogen receptor 1 (Esr1/ERα) signaling is important for memory formation in the hippocampus (Cho et al., 2015). In addition, several Esr1 target genes were downregulated in a mouse model for schizophrenia with working memory deficits (Ouchi et al., 2013). We performed Ingenuity Pathway Analysis (IPA) on DEGs from mutant mice and identified Esr1 as the most prominent upstream regulatory molecule in Ogg1- and/or Mutyh-deficient hippocampus (Figure 4F). The physiological Esr1 ligand β-estradiol and Otx2, an Esr1 downstream target, were also proposed as upstream regulators of the DEGs found in the mutant mice. Strikingly, Esr1 target genes showed significant upregulation in $Ogg1^{-/-}$ and/or $Mutyh^{-/-}$ mice (Figure 4G), suggesting that Ogg1 and Mutyh are involved in repression of Esr1 signaling in the hippocampus to regulate learning and memory.

DISCUSSION

We and others have demonstrated learning and memory defects in mice deficient of Neil1, Neil3, and Ogg1 DNA glycosylases (Liu et al., 2011; Cardozo-Pelaez et al., 2012; Canugovi et al., 2012; Regnell et al., 2012). 5- to 6-month-old Ogg1 KO mice were shown to perform poorly on the rotarod (Liu et al., 2011), and 26-month-old mice displayed spontaneous motor behavior deficiencies, not seen in 3-month-old mice, when subjected to the open field test (Cardozo-Pelaez et al., 2012). Consistent with this, our $Ogg1^{-/-}$ mice showed no activity or movement defects at 4 months of age. However, inactivation of both Ogg1 and Mutyh leads to increased activity, decreased anxiety-like behavior, and impaired learning capability. We have previously observed a similar phenotype in mice deficient of the Neil3 DNA glycosylase (Regnell et al., 2012). Oxidative stress has been implicated in anxiety-related studies, but mainly in relation to the first line of defense (Hovatta and Barlow, 2008). To the best of our knowledge, there are no studies reporting Ogg1 and/or Mutyh (or any other DNA glycosylase) defects in models for anxiety and cognition. Based on our observations, it appears that Ogg1
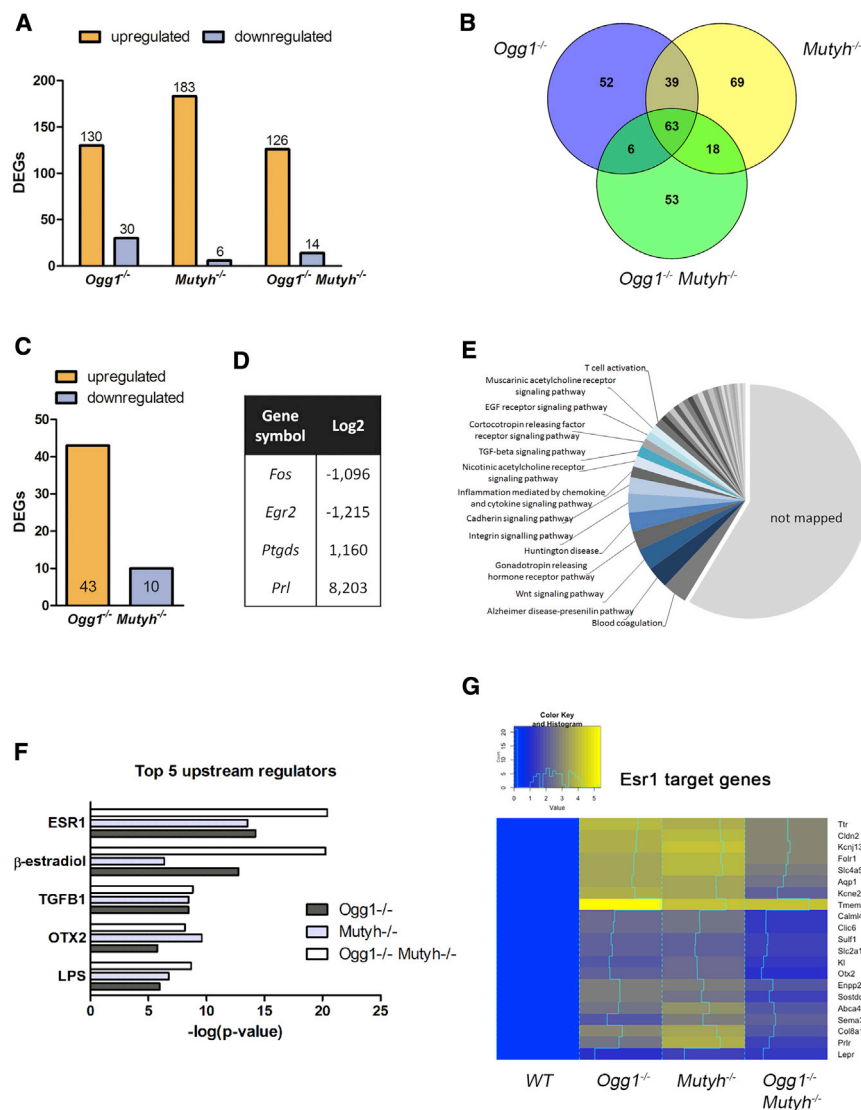
Figure 4. RNA-Sequencing Analysis Revealed Candidate Genes and Pathways Related to Behavior and Cognition as Differentially Regulated in Mutant Mice

(A) Number of differentially expressed genes (DEGs) that were significantly up- and downregulated in $Ogg1^{-/-}$, $Mutyh^{-/-}$, and $Ogg1^{-/-}$ $Mutyh^{-/-}$ mice compared to WT mice.

(B) Venn diagram showing DEGs that overlap for the mutant mice.

(C) Number of uniquely up- and downregulated genes in $Ogg1^{-/-}$ $Mutyh^{-/-}$ mice as compared to WT.

(D) Top candidate DEGs in $Ogg1^{-/-}$ $Mutyh^{-/-}$ involved in anxiety.

(E) Pathway analysis (PANTHER pathway classification system) of DEGs from all mutant mice compared to WT showing pathways associated with learning and memory (blue).

(F) Top five upstream regulators from DEGs of mutant mice as identified by Ingenuity Pathway Analysis (IPA). The p values were calculated using Fisher's exact test.

(G) Heatmap presenting log2 fold change of Esr1 target genes from mutant mice as compared to WT.

sample workup. Thus, 8-oxoG measurements should be interpreted with caution. Nevertheless, it is possible that other activities or pathways (e.g., Mth1 and mismatch repair [MMR]) are more important than Ogg1 and Mutyh to avoid global accumulation of 8-oxoG in hippocampus and hypothalamus (Nakabeppu et al., 2006; Brierley and Martin, 2013). Furthermore, despite similar levels of 8-oxoG in DNA glycosylase-deficient mice, we cannot exclude that an increase in fixation of mutations arising during development may contribute to the phenotype.

RNA-sequencing data revealed that 81%–96% of the DEGs in the hippocampus of $Ogg1^{-/-}$ and/or $Mutyh^{-/-}$ mice were upregulated compared to WT mice. Notably, there was a major overlap between the genotypes. Although unique DEGs in the DKO mice were few, we identified a subset of genes that associates with anxiety-like behavior, supporting that both glycosylases are required to regulate distinct pathways that modulate hippocampal activity (i.e., gonadotropin-releasing hormone receptor and corticotropin-releasing factor receptor signaling pathways). In addition, the hippocampal transcriptome in DNA-repair-deficient mice revealed altered expression of multiple pathways that could contribute to modulation of learning and memory. Recently, Cho and colleagues showed late persistent suppression of Esr1 signaling in hippocampus of mice after contextual fear conditioning (Cho et al., 2015). Notably, we found that Esr1 target genes were upregulated in hippocampus of all three mutants, indicating that Ogg1 and Mutyh may play an important role in repressing Esr1 signaling during memory formation.

and Mutyh modulate anxiety synergistically, whereas Ogg1 seems to have a dominant effect on learning and an antagonistic effect to Mutyh.

Recently, Møller and colleagues compared 69 studies monitoring accumulation of oxidative DNA damage in rodents (Møller et al., 2010). In spite of the overall conclusion stating that 8-oxoG accumulates with age and in glycosylase-deficient animals, several of the studies reported no significant accumulation in brain (Russo et al., 2004; Wong et al., 2006; Fraga et al., 1990; Sai et al., 1992). Accumulation of 8-oxoG has previously been reported in Ogg1 KO mice; however, older mice and other brain regions were analyzed (Cardozo-Pelaez et al., 2012). We observed no apparent differences in 8-oxoG levels in hippocampus and hypothalamus of 6-month-old mice, indicating that global repair of 8-oxoG is not the major function of Ogg1 and Mutyh in these brain regions in adult mice. Notably, measurements of 8-oxodG in mammalian cells and tissues are challenging because of artificial oxidation of guanine in DNA during DNA extraction and

Increasing evidence suggests a role for DNA base damage and DNA glycosylases in regulating cell function and epigenetic changes. Olinsky and coworkers recently reported selective accumulation of 8-oxoG in transcriptionally active chromatin (Zarakowska et al., 2014), and it has been suggested that the BER pathway links hypoxia-induced introduction of oxidative DNA modifications in promoters of hypoxia-inducible genes to transcriptional activation (Pastukh et al., 2015). Cortázar and colleagues showed that the thymine DNA glycosylase (TDG), which specifically recognizes G/T mismatches, maintains epigenetic stability during embryonic development (Cortázar et al., 2011). In addition, several other DNA glycosylases, including Ogg1, have been implicated in active DNA demethylation as a means of gene regulation in adult brain tissue (Spruijt et al., 2013). Moreover, ROS-induced guanine oxidation at CpG dinucleotide sequences interferes with the ability of DNA to function as a substrate for DNA methyltransferases (DNMTs), thereby inhibiting DNA methylation (Weitzman et al., 1994; Valinluck et al., 2004; Maltseva et al., 2009). The BER pathway has been demonstrated to be necessary for histone modification-mediated epigenetic regulation (Perillo et al., 2008). Surprisingly, we identified by gene set enrichment analyses (http://software.broadinstitute.org/gsea/) a significant overlap between the DEGs from KO mice and the Meissner brain high-CpG-density promoters (HCPs) with H3K4Me3 and H3K27Me3 gene set (false discovery rate [FDR]-corrected p value < 1.33E-15). These genes have high-CpG-density promoters bearing histone H3 trimethylation at K4 and trimethylation at K27 and are generally repressed in brain (Meissner et al., 2008). Notably, all overlapping genes showed increased expression in $Ogg1^{-/-}$ and/or $Mutyh^{-/-}$ mice, indicating either a loss of the bivalent repressive histone mark or a change in the underlying CpG methylation pattern. Taken together, these data support that the cognitive phenotypes observed in Ogg1- and/or Mutyh-deficient mice could be a consequence of accumulation of 8-oxoG at gene regulatory regions that may lead to dysregulation of epigenetic and transcriptional states in neuronal cells.

In summary, Ogg1 and/or Mutyh deficiency affects behavior and cognition despite no significant influence on the accumulation of 8-oxoG in brain regions essential for cognition and anxiety. Transcriptome analysis revealed that DEGs within the hippocampus of Ogg1- and/or Mutyh-deficient mice associate with pathways important for anxiety, learning, and memory. Notably, the high percentage of overlap in DEGs between the KO mice suggests that Ogg1 and Mutyh cooperate in regulating gene expression. The molecular mechanisms underlying the observed differences in behavior and cognitive function in Ogg1- and/or Mutyh-deficient mice merit further investigation.

EXPERIMENTAL PROCEDURES

All materials and methods are described in detail in Supplemental Experimental Procedures.

Mice

The $Ogg1^{-/-}$ and $Mutyh^{-/-}$ mice were kind gifts from Arne Klungland (Klungland et al., 1999) and Yusaku Nakabeppu (Sakamoto et al., 2007). They were crossed to obtain $Ogg1^{-/-}Mutyh^{-/-}$ mice. C57BL/6 mice were used as WT controls. 4- to 7-month-old male mice were used, unless otherwise stated.

Open Field Test

The test was conducted in a custom-made white wooden box divided into four arenas (Hall and Bakllchey, 1932). The mice were allowed to explore freely for 45 min.

Elevated Zero Maze

The test was conducted in a custom-made apparatus consisting of a white 5-cm-wide circular runway with four alternating open and closed areas (Shepherd et al., 1994). The mice were allowed 5 min for exploration.

Morris Water Maze

The test was carried out in a white circular pool containing an escape platform at a fixed position (Vorhees and Williams, 2006). During training (days 1–4), the platform was kept 0.5 to 1 cm below the water surface, and during retention tests (days 5 and 12), it was submerged to the bottom of the pool.

LC-MS/MS Quantification of 8-oxodG

DNA was extracted from hippocampus of male and hypothalamus of female mice using a DNeasy Blood and Tissue Kit (QIAGEN, 69506) or an AllPrep DNA/RNA/Protein mini Kit (QIAGEN, 80004), respectively, and subjected to LC-MS/MS.

RNA-Sequencing Analysis

RNA was extracted from hippocampus using an RNeasy Microarray Tissue Mini Kit (QIAGEN, 73304) and sent to BGI Tech Solutions for RNA-sequencing analysis.

Immunohistochemistry and Imaging

Coronal paraffin sections were stained with α-MAP2 (1:8,000; Sigma, M4403) using Dako ARK (Animal Research Kit), peroxidase (Dako, K3954). Results were documented using a Zeiss Axioplan 2 microscope connected to an AxioCamHRc camera. All images were processed and quantified using ImageJ 1.42q software (NIH).

Statistics

Data were analyzed using ANYmaze and R version 3.0.1 and Microsoft Excel. Comparison of means was done with an unpaired, two-tailed t test.

ACCESSION NUMBERS

The accession number for the RNA-sequencing data reported in this paper is GEO: GSE73029.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.001.

AUTHOR CONTRIBUTIONS

M.D.B., G.A.H., K.S., R.S., V.R., and M.B. designed research; M.D.B., G.A.H., K.S., R.S., V.R., A. Kuśnierczyk, C.V.B., and S.V. performed experiments; A. Klungland and Y.N. provided mouse strains; A.D.R. performed statistical analysis of behavioral data; and M.D.B., G.A.H., A.D.R., K.S., V.R., A. Kuśnierczyk, C.V.B., L.E., G.S., Y.N., A. Klungland, T.W.B., and M.B. contributed to interpretation of the results and to writing of the paper.

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Synergistic Actions of Ogg1

and Mutyh DNA Glycosylases

Modulate Anxiety-like Behavior in Mice

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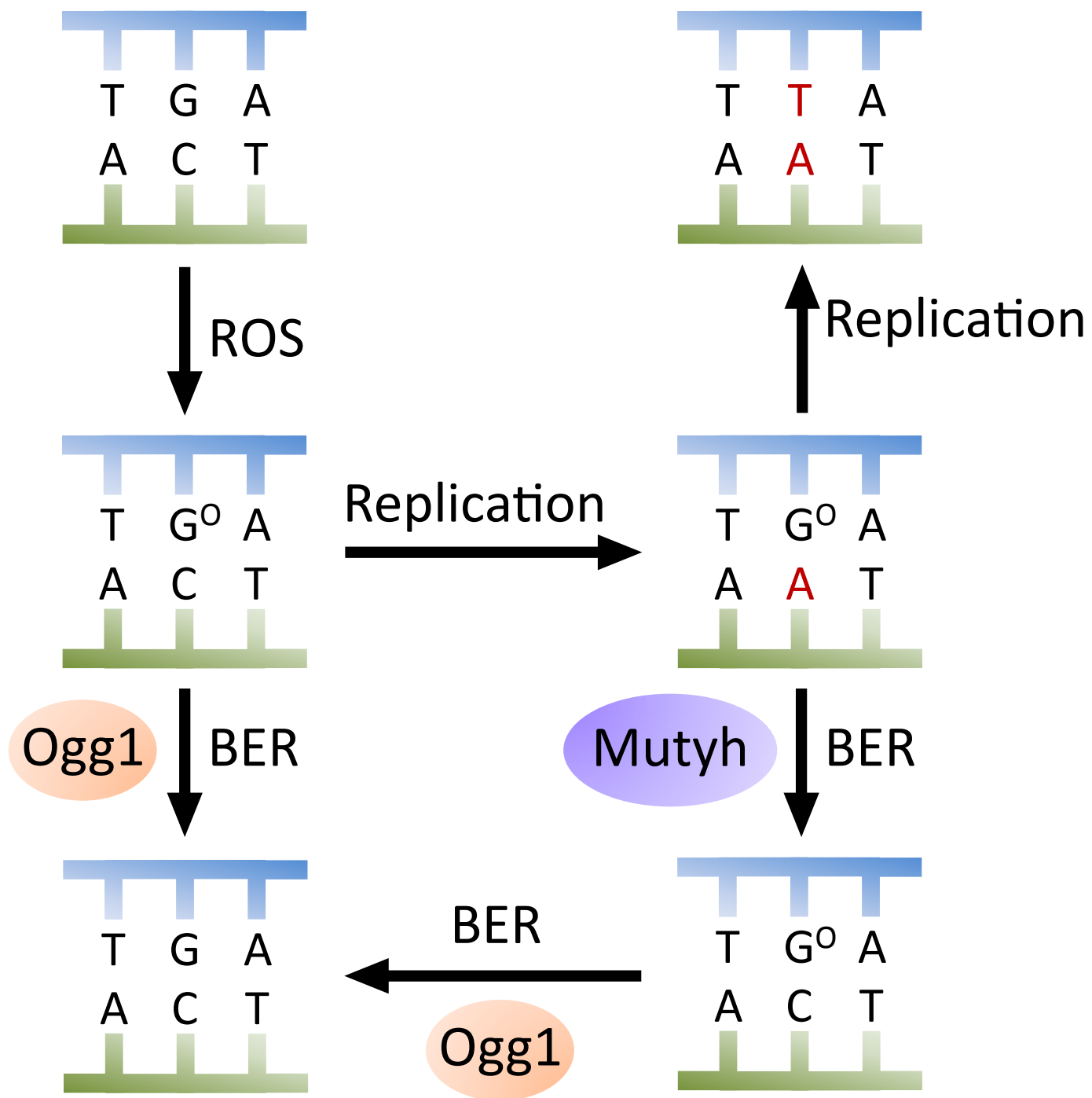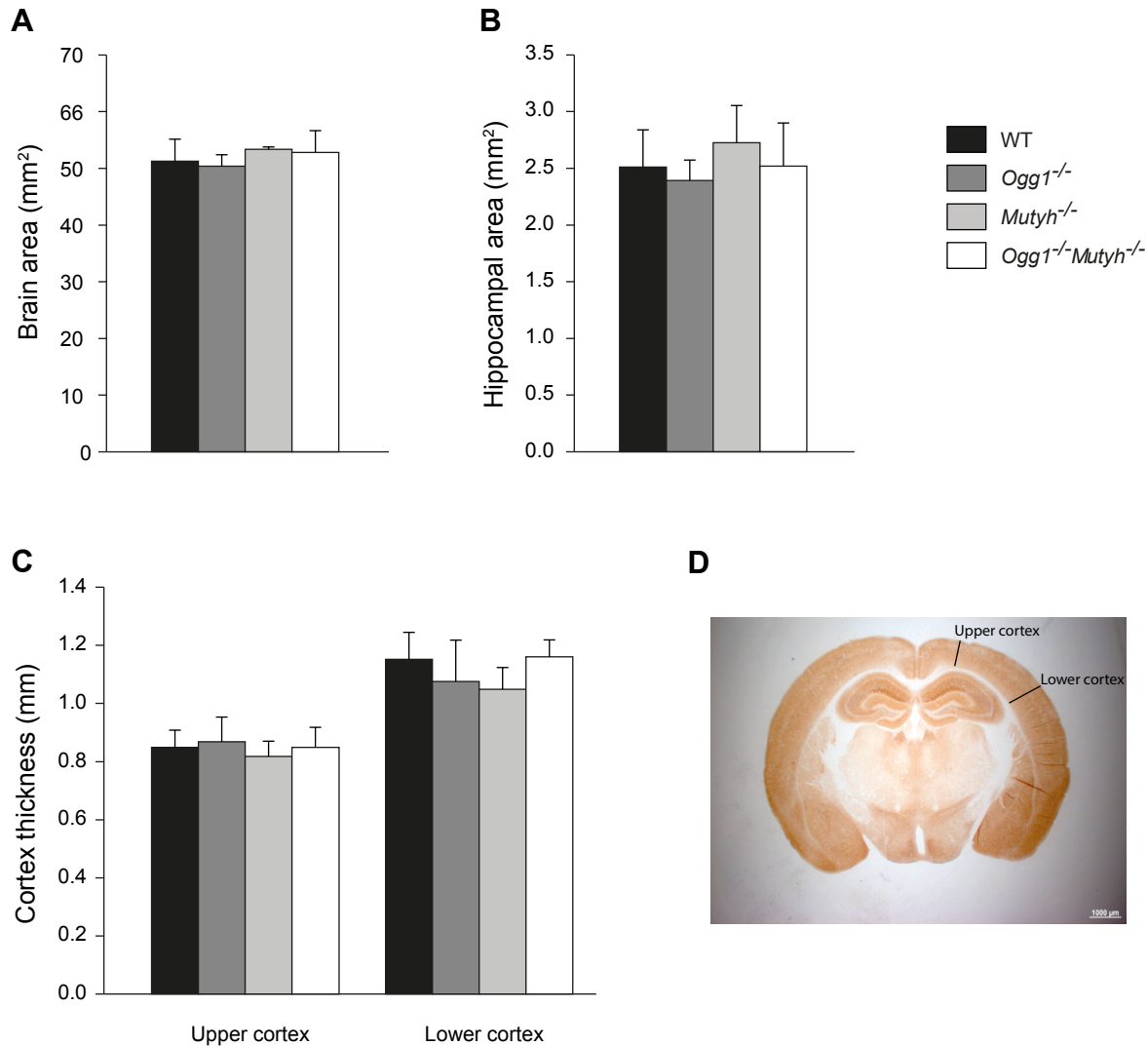
Figure S1

Figure S2

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Repair of 8-oxoG (G^O) resulting from oxidation of G in DNA, related to Introduction. BER initiated by Ogg1 removes the majority of 8-oxoG oxidized in DNA. If 8-oxoG is not removed before DNA replication, replicative DNA polymerases δ and ϵ incorporate dAMP opposite 8-oxoG with a high frequency. The resulting 8-oxoG:A is repaired by Mutyh, so that the the correct base pair is restored and Ogg1 is given a second chance to remove 8-oxoG. However, if the 8-oxoG:A base pair is not repaired before a second round of DNA replication, it leads to an A:T transversion mutation. ROS, Reactive Oxygen Species.

Figure S2. No differences in brain morphology of mice deficient of Ogg1 and/or Mutyh, related to Figures 1 and 2. Coronal sections were stained with α-MAP2 antibody, analyzed by microscopy and photographed. Brain area (A), hippocampal area (B), and cortex thickness (C) were measured by using ImageJ. (D) Representative image. Mean with STD is shown; n = 4-5 mice per genotype. Scale bar: 1000 μM.

EXTENDED EXPERIMENTAL PROCEDURES

Ethics Statement. All experiments were approved by the Norwegian Animal Research Authority (NARA), and conducted in accordance with the laws and regulations controlling experimental procedures in live animals in Norway and the European Union's Directive 86/609/EEC.

Animals. The $Ogg1^{-/-}$ and $Mutyh^{-/-}$ mice were kind gifts from Arne Klungland (Klungland et al., 1999) and Yusaku Nakabeppu (Sakamoto et al., 2007), respectively. They were backcrossed into C57BL/6 for at least 10 generations and crossed with each other to obtain $Ogg1^{-/-}Mutyh^{-/-}$ mice. In all experiments reported, we used adult (4-7 months old) male mice, unless otherwise stated. C57BL/6 mice were used as WT controls. The mice were housed and bred in a 12 hr light/dark cycle at the Department of Comparative Medicine, Oslo University Hospital, Rikshospitalet, Norway, with a diet of pellets and water *ad libitum*.

Behavioral studies. Four months old WT (n=17-22), $Ogg1^{-/-}$ (n=10-11), $Mutyh^{-/-}$ (n=13-14) and $Ogg1^{-/-}Mutyh^{-/-}$ (n=14-18) mice were used in all behavioral experiments presented herein. During testing the researchers were hidden behind a curtain, and the positions of the mice were tracked and stored at 10 Hz by the ANY-maze video tracking system (Stoelting, IL, USA).

Open Field Test. The open field test was conducted in a custom-made white wooden box divided into four arenas (Hall and Ballchey, 1932). Each arena measured H35 cm x L40 cm x W40 cm, and shared walls with two other arenas. An area of L20 cm x W20 cm defined the center area zone of each arena. The light was kept at approximately 110-130 lx in each arena and the mice were placed in their respective arenas and allowed to explore freely for 45 minutes. The mice were tested in groups of four.

Elevated Zero Maze. The zero maze task was conducted in a custom-made apparatus consisting of a white 5-cm wide circular runway with an inner diameter of 50 cm, elevated 65 cm above the floor (Shepherd et al., 1994). The runway had four alternating open and closed areas. The closed areas had metal outer walls and transparent inner walls, which were 20 cm high. 1-cm high lips along the inner and outer edges of the open areas prevented the mice from falling off the runway. The light was kept at approximately 70-90 and 120-140 lx in the closed and open areas, respectively. The mice were placed on the maze facing a closed area and allowed 5 minutes for exploration. An open area entry was defined as 85% of the mouse being inside an open area.

Morris Water Maze. The MWM task was performed in a white circular pool, 120 cm in diameter and 30 cm deep, filled 2/3 with white, opaque water (SikaLatex liquid, Sika, Norway or Flutex 7S paint, Flügger, Norway) (Vorhees et al., 2006). The temperature was kept at 23 ± 1 °C and the light at approximately 70 lx. The pool was virtually divided into four quadrants (north/target, south, east and west) using the ANYmaze software. An escape platform, 11 cm in diameter, was located at a fixed position halfway between the midpoint of the pool and the wall in the north/target quadrant. During training trials the platform was kept 0.5-1 cm below the water surface. The mice were trained for four days with two blocks of four training trials each day. The four trials in each block were conducted consecutively for each mouse and the mice were released into the pool facing the wall at four fixed positions (NE, SE, SW and NW) along the pool wall in a pseudorandom order. The mice were given a maximum of 60 sec to locate the hidden escape platform. Whether or not the mice found the platform, they were placed on the platform and allowed to sit there for 15 sec during the inter-trial interval. The mice were allowed to rest for 3-4 hours between

the two blocks. On days 5 and 12, a single retention trial of 60 sec was performed for each mouse, during which the escape platform was submerged to the bottom of the pool and the mice were released from position south (S).

DNA extraction. Total DNA was extracted from hippocampi of 6 months old male mice (mice from behavior studies and naïve mice) by using DNeasy Blood and Tissue Kit (Qiagen, 69506) and from hypothalamus of 6-7 months old female mice (naïve) by using AllPrep DNA/RNA/Protein mini Kit (Qiagen, 80004), according to manufacturer's protocol. Frozen tissue was transferred to either Lysing matrix D vials (MP Biomedicals, 6913-050) containing 180 μl buffer ATL (kit 69506) or tubes with stainless steel beads containing 350 ml buffer RLT (kit 80004) and homogenized using a FastPrep®-24 instrument (MP Biomedicals), 2 x 15-20 s, 5 min on ice between runs. Samples were further processed according to the respective protocols. DNA concentrations were measured by using an ND-1000 spectrophotometer (Nanodrop Technologies, Sweden).

LC-MS/MS analysis of 8-oxodG in genomic DNA. 2 μg of genomic DNA was enzymatically hydrolyzed to deoxyribonucleosides by DNase I (Roche, 04716728001), nuclease P1 from *Penicillium citrinum* (Sigma, N8630), and alkaline phosphatase from *E. coli* (Sigma Aldrich, P5931) in 10 mM ammonium acetate, pH 5.3 at 40 °C for 30 min. Three volume equivalents of ice-cold methanol were added to precipitate contaminants. Following centrifugation at $16000 \times g$ at 4 °C for 30 min the supernatants were dried under vacuum at room temperature. The resulting residues were dissolved in 50 μl of water for LC-MS/MS quantification of 8-oxodeoxyguanine (8-oxodG). A portion of each sample was diluted for the quantification of unmodified deoxynucleosides dA, dC, dG, and dT. Chromatographic separation of nucleosides was performed using a Shimadzu Prominence LC-20AD HPLC system with an

Ascentis Express C18 2.7 μm 150 x 2.1 mm i.d. column equipped with an Ascentis Express Cartridge Guard Column (Supelco Analytical, Bellefonte, PA, USA) with EXP Titanium Hybrid Ferrule (Optimize Technologies Inc.) at a flow rate of 0.14 ml/min at ambient temperature. The mobile phase consisted of A (0.1 % formic acid in water) and B (0.1 % formic acid in methanol). The following conditions were employed during chromatographic separation: unmodified nucleosides – starting with 90% A and 10% B for 0.1 min, followed by a 2.4 min linear gradient of 10-60% B, and 4.5 min re-equilibration with the initial mobile phase conditions; 8-oxodG – starting with 95% A and 5% B for 0.5 min, followed by a 7.5 min linear gradient of 5 - 45% B, and 5.5 min re-equilibration with the initial mobile phase conditions. Online mass 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 mass transitions 252.1→136.1 (dA), 228.1 → 112.1 (dC), 268.1→152.1 (dG), 243.1→127.1 (dT), 284.1→168.1 (8-oxodG).

RNA extraction. Total RNA was extracted from hippocampi of 6-7 months old naïve mice by using RNeasy Microarray Tissue Mini Kit (Qiagen, 73304) according to manufacturer's protocol. Frozen tissue was transferred to Lysing matrix D vials (MP Biomedicals, 6913-050) containing 1 ml of QIAzol Lysis Reagent and homogenized by using a FastPrep®-24 instrument (MP Biomedicals), 2 x 25 s, 5 min on ice between runs. On-column DNase digestion was performed as described. DNA was eluted in 2 x 40 μl of RNase-free water in one collection tube. RNA concentrations were measured by using an ND-1000 spectrophotometer (Nanodrop Technologies, Sweden) and RNA quality was examined by using Agilent RNA 6000 Nano Kit (Agilent Technologies,

5067-1511) in combination with a 2100 Bioanalyzer (Agilent Technologies) according to manufacturer's protocol.

RNA sequencing analysis. Three RNA samples from each genotype (all males, except for two *Mutyh-/-* females) were pooled and sent to BGI tech solutions, China, for RNA sequencing analysis. Library construction, sequencing (Illumina HiSeq2000) and the main part of the bioinformatics analysis were performed at BGI. An absolute value of log2Ratio $\geq$ 1 and false discovery rate (FDR) $\leq$ 0.001 were used to judge the significance of gene expression differences between the WT and the KO mice. The data were further filtered by removing transcripts (common to both WT and KO) with reads per kilobase exon per million reads (RPKM) < 1. Differentially expressed genes (DEGs) were further analyzed by using the PANTHER Pathway classification system (Mi and Thomas, 2009). To identify upstream regulatory molecules, Ingenuity Pathway Analysis (IPA) was applied. The RNA sequence data from this study have been submitted to the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE73029.

Immunohistochemistry and imaging. Mice were deeply anesthetized with a single dose fentanyl/fluanisone+midazolam s.c. and transcardially perfused with PBS followed by cold 4% paraformaldehyde (PFA). Brains were removed and immersion fixed in 4% PFA for at least 24h, dehydrated and embedded in paraffin. To visualize the hippocampi, 4μm thick coronal sections were obtained between bregma -1.2 and -1.3 mm. Before immunostaining, the sections were deparaffinized and rehydrated by incubation at 56 ^{o}C for 10-20 min followed by step-wise incubation in Clear-Rite 3 (Richard-Allan scientific, 6901) for 5 min x 2, 100% ethanol for 1 min x 2, 96% ethanol for 1 min, 70% ethanol for 1 min and water of gradually increased temperature. Antigen retrieval was performed by microwave heating in citrate buffer,

pH 6.0, for 15 min. Slides were cooled at room temperature for 10-20 min and subjected to various staining procedures. Staining with α-MAP2 (1:8000; Sigma, M4403) was performed by using Dako ARK (Animal Research Kit), peroxidase (Dako, K3954) according to manufacturer's protocol. Slides were mounted with Cytoseal XYL (Richard Allen Scientific, R-AS 8312-4). Results were documented by using a Zeiss Axioplan 2 microscope connected to an AxioCamHRc camera. All images were processed and quantified using ImageJ 1.42q software (NIH).

Statistics. Behavioural data were analyzed by using ANYmaze and R version 3.0.1. Statistical analysis of other data was done with Microsoft Excel. Comparison of means was done with unpaired, two-tailed t-test. The sum of the time used for all learning trials per mouse each day (max 480 sec) was used to measure performance over the four training days in the Morris Water Maze. Since right-censored (unsuccessful trial completion) data are not adequately accounted for by these values, a non-parametric pairwise Wilcoxon rank-sum test was used to examine the difference between learning times for each genotype (Figure 2A). During probe tests, all genotypes displayed an unexplained tendency to prefer the eastern quadrant over the western (Figure 2C). To quantify any tendency to prefer the target quadrant over the other quadrants we chose to measure the proportion of swimming time spent in the target quadrant by each mouse. This showed a significant dependence upon the day of testing ($p<0.026$) and genotype (*Mutyh$^{-/-}$* showed significantly higher preference over all other genotypes $p < 0.0011$, Tukey multiple comparisons of means). Including a simple interaction term for Day and Genotype shows *Mutyh$^{-/-}$* preference on day 5 to be significantly higher than all other genotypes on day 5 and Mutyh preference on day 12 to be weakly significantly higher than all other day 12 genotypes. The clear conclusion is that *Mutyh$^{-/-}$* mice on average prefer the target quadrant more than all

other genotypes and do not appear to swim as randomly on day 12 as the other genotypes.

SUPPLEMENTAL REFERENCE

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