博士論文（要約）

# Zone specific injury of the olfactory epithelium under dietary restriction and voluntary exercise

（長期の食事制限や自発運動によって引き起こされる

嗅上皮での領域特異的な障害）

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Summary text of Dissertation

# Zone specific injury of the olfactory epithelium under dietary restriction and voluntary exercise

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Oxidative stress causes tissue damage and affects age-related pathologies. Exercise (Ex), protein restriction (PR), and caloric restriction (CR) are robust interventions that reduce oxidative stress and have positive effects on individual organs. However, it remains unknown whether such interventions have similar effects on the olfactory system. I investigated whether 10 months of voluntary Ex, PR, or CR affects cell dynamics in the olfactory epithelium (OE) in male C57BL/6 mice. Diets varied only in their protein-to-carbohydrate ratio: 30% casein reduction (PR diet); 20% caloric reduction (CR diet). I first tested the effectiveness of our Ex, PR, and CR regimes to generate such changes. Then I sought to explore the histological and functional effects of long-term Ex, PR, and CR on cell dynamics in the OE.

**Ex, PR, and CR induce a reduction in the number of OSNs in the DM region of the OE**

I compared coronal sections of the OE from 12-month-old control mice and from 2-month-old mice that had not undergone any experimental regime. The results indicate that 10 mo of control diet does not induce any histological changes in the OE.

Next, I examined whether 10 mo of Ex, PR, or CR affects the number of OSNs in the OE. The number of OSNs and OMP-positive cells in the DM region was significantly decreased in Ex, PR, and CR mice compared with age-matched controls. In the DL, VM, and VL regions, however, I did not observe significant differences between the experimental and control groups. These results indicate that Ex, PR, and CR induce histological changes selectively in the DM region of the OE.

The OE is subdivided into dorsal and ventral zones based on the differential expression of specific types of odorant receptors and endocellular molecules. Because the dorsal zone is distributed in the DM region

of the OE and OSNs in the dorsal zone express NQO1, I hypothesized that Ex, PR, or CR induces histological changes selectively in the dorsal zone and selectively affects NQO1-expressing cells.

I first examined whether the DM region of the OE that showed histological change corresponds to the dorsal zone. The number of NQO1-positive cells in the dorsal zone decreased in Ex, CR, and PR mice compared with age-matched control mice. Furthermore, I found OMP staining was significantly lower in the NQO1-positive square than in the NQO1-negative square in Ex, PR, and CR mice. These results indicate that 10 mo of Ex, PR, or CR can induce damage in the dorsal zone of the OE.

Newly generated OSNs project axons through the cribriform plate to the surface of the OB. I measured the size of the OMP-stained area within individual glomeruli of the OB in Ex, PR, and CR mice. I observed a significant reduction in the size of the OMP-stained area in NQO1-positive OB in Ex, PR, and CR mice compared with age-matched control mice. I did not detect any differences in the NQO1-negative OB. These results suggest that the experimental interventions resulted in the projection of fewer axons from OSNs in the dorsal zone.

To examine whether dorsal-zone-specific injury to the OE functionally disrupts stable sensory inputs to the OB, I measured the expression of the neural activity marker c-fos in NQO1-positive and -negative areas of the OB in Ex, PR, and CR mice after odor stimulation. I observed a significant reduction in the number of c-fos-positive cells in the NQO1-positive area of the OB in Ex, PR, and CR mice compared with age-matched control mice. I did not detect any significant differences in the number of c-fos-positive cells in the NQO1-negative area of the OB in Ex, PR, or CR mice relative to their age-matched controls. These results suggest that the decrease in dorsal-zone OMP-positive cells and OSNs and their axonal projections to the glomeruli is associated with a decrease in the glomerular response to odorants.

**Long-term Ex, PR, or CR is required for dorsal-zone-specific injury**

To examine whether short-term Ex, PR, or CR can induce dorsal-zone-specific injury in the OE, I assessed histological changes in the OE after 4 mo of Ex, PR, or CR. I did not observe significant differences in the number of OSNs or OMP-positive cells in any of the four areas of the OE in Ex, PR, or CR mice compared with age-matched control mice. The results indicate that a period longer than 4 mo is required to induce histological changes in the dorsal zone of the OE.

**Ex, PR, and CR increase proliferation and apoptosis in OSNs**

To examine the mechanism underlying the reduction in OSNs in the dorsal zone of the OE following Ex,

PR, or CR, I examined the number of OE cells that were positive for Ki67 and caspase-3. The number of dorsal-zone Ki67-positive cells and caspase-3 was significantly higher in Ex, PR, and CR mice than in age-matched controls. However, there were no significant increases in proliferation and caspase-3 staining in the ventral zone. These results indicate that Ex, PR, and CR increase the mitosis of progenitor basal cells but that the recovery of cell numbers is incomplete because of an increase in apoptotic OSNs in the dorsal zone.

**Oxidative stress in dorsal-zone OSNs is strongly linked to NQO1 activity**

NQO1, a cytosolic flavoenzyme, protects against oxidative stress through the removal of quinones and other xenobiotic metabolic products. However, NQO1 can also mediate ROS generation via conjugation with SOD, leading to enhanced oxidative stress. I hypothesized that increased ROS generation, mediated by NQO1 activity, induced oxidative stress in this study, resulting in increased apoptosis in the dorsal zone of the OE. I examined the expression of 8-OHdG in Ex, PR, and CR mice as a marker of oxidative stress. I observed a significant increase in the number of 8-OHdG-positive cells in the dorsal zone in Ex, PR, and CR mice compared with age-matched controls, but no such increase in the ventral zone. Furthermore, I co-labeled OSNs with anti-NQO1 and anti-8-OHdG to examine the role of oxidative stress in NQO1 expression. A significant number of 8-OHdG-positive cells were co-labeled with NQO1. These results indicate that increased oxidative stress is present within NQO1-positive cells.

To examine whether increased oxidative stress in the dorsal zone could induce apoptosis of OSNs, I co-labeled OSNs with anti-caspase-3 and anti-8-OHdG. I found that a significant number of 8-OHdG-positive cells were co-labeled with anti-caspase-3. These results indicate that increased oxidative stress induced apoptosis of the OSNs in the dorsal zone in Ex, PR, and CR mice.

Under my hypothesis, ROS generation, mediated by NQO1 activity, should lead to increased SOD activity in the dorsal zone. Mitochondria are a main source of ROS; therefore, I measured the intensity of anti-MnSOD immunostaining to quantify MnSOD activity. The results revealed a significant increase in staining intensity in each condition compared with age-matched control mice. These results indicate that enhanced oxidative stress in Ex, PR, and CR mice is involved in the bioactivation of NQO1 under enhanced MnSOD activity, leading to prominent cell death of OSNs in the dorsal zone.

The present findings show that Ex, PR, and CR induce negative structural and functional effects in the

dorsal zone of the OE and that these changes are associated with NQO1 bioactivation.

Voluntary long-term Ex, PR, and CR can induce changes in the AMPK, SIRT1, mTOR, GH/insulin/IGF1, and FGF21 pathways. These pathways respond to cellular bioenergetics or to sugars and amino acids. I predicted that Ex, PR, and CR would act to reduce anabolic responses and oxidative stress; however, I found instead a significant increase in immunoreactivity for 8-OHdG in the dorsal zone of the OE. I did not see a significant increase in 8-OHdG in the ventral zone, suggesting that different cellular mechanisms regulate the generation of oxidative stress in dorsal and ventral zones of the OE. The preferential generation of oxidative stress in the dorsal zone could be associated with NQO1 activity conjugated with MnSOD, because I found significant co-localization of oxidative stress with NQO1 expression and markers of apoptosis.

NQO1 protects cells against quinone toxicity; however, NQO1 bioactivation within metabolic pathways involving multiple enzymes is not simple. In particular, the conjugation of NQO1 with MnSOD facilitates the generation of ROS through the reaction of unstable hydroquinones with oxygen, leading to oxidative stress. I hypothesize that the physiological properties of NQO1 under enhanced MnSOD activity may cause OSNs to become highly susceptible to damage from environmental quinone agents or endogenous neurotoxins. MnSOD does have antioxidant enzymatic properties; however, an imbalance between ROS generation and antioxidant defenses within the dorsal zone might induce continuous oxidative damage. This would result in structural and functional damage to dorsal-zone OSNs despite the generation of new neurons. Pathological conditions, such as intraperitoneal injection of olfactory toxins and inhalation of hydrogen sulfide, induce localized neural degeneration of OSNs in the dorsal zone. Altogether, these data suggest that NQO1 activity increases OSN susceptibility to the harmful action of other substrates.

It has been argued that aging increases the activity of MnSOD in the brains of rodents. I speculate that the OE in older mice may show enhanced MnSOD activity and NQO1-dependent damage in the dorsal zone in mice with a non-restricted diet. Further studies are needed to clarify the detailed physiological properties of NQO1 conjugated with MnSOD during aging and other pathological conditions.

Other possible mechanisms of dorsal-zone-specific damage could include alterations in internal hormonal signaling, because nutritional status dynamically and profoundly impacts olfactory sensitivity. The OE and OB express high levels of mRNAs for anorexia-signaling hormone receptors, such as leptin, insulin, and IGF1. Metabolic hormones can affect neuron survival and their activity in olfactory networks when

targeting their receptors. Insulin can increase the number of cultured OSNs *in vitro* and prevents the apoptosis of OSNs via activation of intracellular cAMP in adult rats after injury. There may be a decrease in systemic insulin/IGF1 signaling under Ex, PR, and CR conditions in this study that affects the regulation of cell dynamics in the OE. I hypothesize that the high susceptibility of dorsal-zone OSNs to damage is facilitated by a decrease in insulin/IGF1 signaling.

OSNs in the dorsal zone project to the dorsal domain in the main OB. Many mitral cells in the dorsal domain further project to the cortical amygdala, and mediate aversive behavior to spoiled odors and fear responses to predator odors. OSNs in the ventral zone project to the ventral domain in the main OB. Mitral cells located in the posteroventral part of the ventral domain project to the anterior medial amygdala, and mediate attractive social behaviors such as mating and conspecific social odor recognition. Selective reduction of OSNs in the dorsal zone under long-term Ex, CR, and PR interventions is expected to decrease sensitivity to odors that activate the dorsal domain stream, and thus increase the odor threshold for the induction of negatively motivated behaviors, such as aversive behavior and fear responses. By contrast, long-term Ex, CR, and PR had little effect on OSNs in the ventral zone, suggesting that these interventions would not change the odor threshold for the induction of positively motivated behaviors, such as attractive social behaviors. Current study results suggest that compared with domesticated conditions, wild conditions with Ex and moderate CR and PR would result in decreased sensitivity of the dorsal domain stream to aversive odors but would not change the sensitivity of the ventral domain stream to attractive odors.