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STUDY OF NEW MOLECULAR TARGETS IN PRECLINICAL MODELS OF OBESITY

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1. Summary of the project

The consumption of highly palatable and highly caloric food could produce an altered functionality of dopamine type-2 receptor (D2) striatopallidal medium spiny neurons (MSNs), leading to the development of obesity through a mechanism that involves the dual specificity tyrosine-phosphorylation-regulated kinase 1A (Dyrk1A) activities. The general aim of this research proposal was to evaluate the impact of overconsumption of highly palatable and highly caloric food on striatopallidal MSNs activity modifications to characterize the cellular and molecular mechanisms underlying such alterations. Particular interest was focused on the role of Dyrk1A in these phenotypes. Thus, striatopallidal-specific profiling of mRNAs following an overeating-induced obesity model and an evaluation of the functional implication of identified gene candidates was performed. A novel preclinical mouse model for the study of obesity targeting MSNs, D2-RiboTag mice was generated. The pathogenic features of obesity were studied in these mice employing a behavioral model of eating disorder, namely overeating behavior by ad lib exposure to high palatable and caloric food. The actively translated mRNAs in neurons expressing D2 receptors from the dorsal and ventral striatum were studied by RNAseq in this behavioral model. Relevant modified mRNAs were identified by bioinformatics analysis to find out new potential therapeutic targets for the treatment of obesity. Posterior validation of the candidate genes was performed to ascertain that the candidate genes were functionally relevant. Finally, a validation of the new potential therapeutic targets for the treatment of obesity was carried out.

2. Results obtained

In this project, we studied the neurobiological mechanisms involved in overeating leading to obesity. We obtained behavioral signatures of obesity development and loss of control over food intake. We developed obesity animal models in an inbred strain of mice with C57Bl6/J background. This aim allowed us to have the genetic variable under control and study the specific molecular and genetic targets in detail. In these models, we exposed mice to highly palatable food with different regimes of access to food for a long time. We observed alterations at peripheral metabolic parameters and at the molecular and cellular brain level that validated the obesity mice model. Our results suggested that the *Dyrk1A* gene could be essential as a modulator of eating behavior

after consuming palatable foods. We had analyzed the development of obesity and the feeding behavior induced by the intake of high caloric-palatable diets in different murine models of *Dyrk1A*, a model overexpressing *Dyrk1A*, and a model with reduced expression of *Dyrk1A* in dopamine D2 receptor-expressing neurons. We have characterized a mouse model with partial reduction of *Dyrk1A* expression, the *Dyrk1A* heterozygous model, demonstrating the crucial involvement of this gene in feeding behavior. Also, the transcription and translation profiles of D2 receptor-positive cells, evaluated by high-throughput RNAseq with labeled ribosome-bound mRNAs, allowed us to identify 2928 genes encoding proteins significantly enriched in the nucleus accumbens (NAc). Among them, the *Wfs1* gene was one of the most statistically significant of the RNAseq analysis.

Furthermore, we investigated by transcriptomic analysis the functional alterations that specifically occurred in the neuronal subpopulations of D2-positive neurons during the development of compulsive-like behavior. For this purpose, we took advantage of D2-RiboTag mice, an innovative transgenic mouse line, allowing the selective expression of hemagglutinin (HA) tag in D2 receptor-containing cells. The selected genes for pharmacological functional validation were *Adora2a* (A2a receptor), *Cholecystokinin* (CCK), and *DAGLα.* Comparing the transcriptomic profiles of D2 receptor-positive cells in the NAc among binge, free choice, and control standard diet groups revealed an upregulation of the *Adora2a, CCK, and DAGLα* genes in mice of the binge eating group compared to free choice and control standard diet groups. Pharmacological validation of the selected candidate genes was performed by the local administration of the CCK agonist CCK-8S (vehicle, 1, 2, or 5 ng/side), the DAGLα inhibitor O-7460 (vehicle, 1, 2, or 10 ng/side), and the A_{2a} agonist CGS 21680 (vehicle, 1, 2 or 4 ng/side) bilaterally into the NAc of mice exposed to the binge eating protocol or the standard diet. Concerning the administration of the CCK agonist CCK-8S, results showed that the CCK receptor activation did not modify either food intake or locomotor activity. Results of the pharmacological validation with the DAGLα inhibitor O-7460 (vehicle, 1, 2, or 10 ng/side) showed that only the highest dose of 10 ng/side was able to decrease the chocolate intake in the binge eating group after 1.5h of diet exposure without affecting locomotor activity. Finally, the pharmacological validation was performed by injecting the A2a receptor agonist CGS 21680 (vehicle, 1, 2, or 4 ng/side) bilaterally into the NAc of mice exposed to the binge eating protocol or the standard diet administration and locomotor activity was also evaluated. Results showed that all doses tested

produced a trend or a significant decrease of chocolate food intake at 1.5h, 3h, and 24h after binge eating diet exposure compared to basal levels. With the highest dose of 4 ng/side, locomotor activity was also decreased during the first 60 minutes after intra NAc administration.

In summary, intrastructural pharmacological targeting into the NAc of A2a receptor and DAGLα enzyme by activation/inhibition, respectively, may regulate compulsive-like behavior by food intake reduction, specifically high energy-dense palatable food. For the functional validation using a genetic approach, the *Adora2a* gene was selected. A viral vector approach was used to deeply explore this candidate gene regulatory role in D2 receptor-expressing neurons into the NAc in compulsive-like behavior development. Previous studies have shown that A2a activation and D2-like receptor blockade counteract cocaine and food relapse. It is proposed that A2a receptor- and D2 receptor-mediated adenosine and dopamine signaling antagonistically interact in the striatopallidal GABAergic neurons to regulate cocaine and food-seeking behavior. Taking into account that our results demonstrated that the intrastructural pharmacological activation of the A2a receptor decreased compulsive-like behavior by highly caloric and palatable food intake reduction, we selected this candidate gene to perform a functional genetic validation using a viral vector approach. Specifically, we tested whether the selective overexpression of *Adora2a* in the NAc core-ventral pallidum (VP) projections induced a decreased chocolate intake and reduced seeking for palatable food self-administration. Using these experimental conditions, we aimed at mimicking the upregulation of the *Adora2a* gene observed in binge-eating mice according to RNA-seq results. We hypothesized that this upregulation may be produced to reduce excessive food intake and can represent a protective mechanism. For specific overexpression of *Adora2a* in NAc core-VP projections, we used a dual viral vector approach with a Cre-dependent AAV- Adora2a (AAV-hSyn-DIO-Adora2a-mCherry and AAV-control (AAV-Syn1-DIO-mCherry) viral vector injected into the NAc core, and an AAV- retrograde-Cre (AAV-pmSyn-GFP-Cre) injected into the VP. We first verified the AAV injection site by autofluorescence visualization of mCherry and Cre recombinase. To test the effect of *Adora2a* overexpression in chocolate food taking and food-seeking, an experimental protocol of 20 weeks that combined binge eating and operant behavior was applied.

Results showed that mice that overexpressed the *Adora2a* gene in the projections from the NAc core to the VP and were exposed to the binge diet reduced the consumption of chocolate across binge eating cycles compared to basal levels and compared to the control group. This result suggests that the overexpression of the *Adora2a* gene was a protective factor related to obesity development, as predicted. Next, the motivation for chocolate-flavored pellets was reduced in mice overexpressing the *Adora2a* gene in the NAc core to VP network independently of the diet. No differences in persistence to response or compulsivity were obtained depending on the AAV injection or exposure diet. Impulsivity-like behavior was also measured, and mice overexpressing *Adora2a* exposed to a standard diet showed reduced impulsivity-like behavior. In the reversal test, mice were tested for cognitive flexibility, and the active and inactive levers were reversed. Results showed that mice from the binge group were less flexible, and there was a slight trend of mice with a binge eating diet and AAV-*Adora2a* injection to be more flexible than the control group. The three criteria of food addiction (motivation, persistence to response, and compulsivity) were used to classify mice as vulnerable or resilient to develop compulsive-like behavior and loss of control over food intake. According to the behavioral tests used to measure the food addiction-like behavior, mice were categorized as vulnerable or non-vulnerable animals depending on the number of positive criteria they had achieved. An animal was considered positive for an addiction-like criterion when the specific behavioral test score was above the 75th percentile of the normal distribution of the chocolate control group. Mice that achieved two or three addiction-like criteria were considered vulnerable animals, and mice with 0 or 1 addiction-like criteria were considered non-vulnerable animals. The percentage of mice classified as vulnerable was 0% in mice overexpressing *Adora2a* of the standard diet group. This percentage was much lower than the expected 20% of vulnerable mice found in the standard control group.

In contrast, in the group of binge mice, overexpressing *Adora2a* did not reduce the percentage of mice that meet the vulnerability criteria. This percentage was not augmented for the binge diet. In conclusion, results validated the previous findings with pharmacological validation and pointed out a protective role of the *Adora2a* overexpression in the NAc to VT projecting neurons for the compulsive-like behavior leading to obesity development. No significant differences in emotional or cognitive behavior were obtained in depression or anxiety measured in the forced swimming or elevated plus maze tests. The diet or AAV injection effects were evaluated in the novel

object recognition test, and no differences were observed in short or long-term memory. Finally, no significant differences were obtained in rearings or locomotor activity at pre- or post-surgery, suggesting that the viral approach did not affect activity-like behavior.

In summary, we elucidated the crucial role of the NAc core-VP pathway modulated by *Adora2a* as a critical mechanism for motivation for chocolate. Overexpression of the *Adora2a* gene in this pathway plays a crucial role in preventing these phenotypes. Our results provided new mechanistic approaches that might be valued for establishing new therapeutical perspectives for treating and preventing overweight and obesity. In addition, our findings shed new light on prevention measures by identifying neural mechanisms required for strengthening the resilient phenotype that could potentially prevent obesity development.

3. Relevance with possible future implications

We elucidated the NAc core-VP pathway's critical role modulated by *Adora2a* as a critical mechanism for motivation for palatable food-seeking and chocolate overconsumption. Overexpression of the *Adora2a* gene in this pathway plays a crucial role in preventing these phenotypes. Thus, our results provided new mechanistic approaches that might be valued for establishing new therapeutical perspectives for treating and preventing overweight and obesity at the clinical level. In addition, our findings shed new light on prevention measures by identifying neural mechanisms required for strengthening the resilient phenotype that could potentially prevent obesity development. Our results also suggest that the *Dyrk1A* gene could be essential as a modulator of eating behavior after consuming palatable foods. Analysis of the transcription and translation profiles of D2 receptor-positive cells, evaluated by high-throughput RNAseq with labeled ribosome-bound mRNAs, allowed us to identify 2928 genes encoding proteins significantly enriched in the NAc. Among them, the *Wfs1* gene was one of the most statistically significant of the RNAseq analysis. We have elucidated the crucial role of the pathway comprising the NAc core and VP modulated by the *Adora2a* gene as a critical motivational mechanism for searching for highly palatable food and uncontrolled chocolate consumption. Overexpression of the *Adora2a* gene in this pathway plays a crucial role in the prevention of these phenotypes.

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