**Scientific Abstract:** **Deciphering the cellular and molecular mechanisms in the brain underlying the interaction between high-fat diet consumption and social isolation**

 Social isolation imposes a severe mental and physiological burden on humans. During the COVID-19 pandemic, social isolation has emerged as a major risk factor for the development of psychiatric and emotional disorders. Stress and social isolation promote palatable food intake in all tested mammals as a coping mechanism that limits stress responses. The mechanisms underlying this link, however, remain unknown. **While prior studies have focused on adults, the impact of social isolation on children and adolescents is even more profound and remains under-studied.**

 Prior efforts to reverse social isolation-induced deficits through social regrouping or neuronal manipulations achieved only transient effects. Our preliminary findings in **juvenile and adult** animals revealed that both high-fat diet (HFD) consumption and social isolation, when provided separately, led to **age-dependent** deficits in social memory and prefrontal plasticity. Unexpectedly, however, the combination of both of these treatments resulted in the **age-dependent** rescue of these deficits, which could persist for one month following the treatment of juvenile animals. At the transcriptomic level, several microRNAs (miRNAs) and their mRNA targets were found to modify cognitive performance through their context-dependent impacts on cellular and subcellular functions, highlighting them as putative candidate regulators of the molecular basis for this striking phenotype. In this proposal, we seek to explore the behavioral, cellular, and molecular changes in a specific brain circuit consisting of the amygdala, hippocampus, and prefrontal cortex in an effort to understand how individual factors and interactions among these factors ultimately contribute to the observed rescue effect. ***We*** ***hypothesize that HFD and social isolation generate separate overlapping signatures at the cellular and molecular levels and that the combination of these treatments results in unique molecular reprogramming mediated by differential miRNA expression. We further hypothesize that*** **age*- and brain region-specific reprogramming can have an enduring effect on social memory and neuronal plasticity.*** We will examine both age- and sex-dependent differences in these responses, and we will conduct transcriptomic and proteomic analyses to identify putative miRNA-regulated genes and pathways underlying the phenotypes associated with HFD, social isolation, or the combination of the two before ultimately inhibiting/activating miRNAs to test their functional roles.

This project provides a unique experimental setting to understand the behavioral, cellular, and molecular signatures associated with detrimental social isolation and unhealthy food intake while allowing for the identification of the mechanisms underlying the rescue effect when the two are combined. These findings may aid future efforts to alleviate social impairment, particularly in non-obese children, through the development of personalized diets containing appropriate micronutrients necessary to prevent obesity while buffering the detrimental impact of social isolation.

Deciphering the cellular and molecular mechanisms in the brain underlying the interaction between high-fat diet consumption and social isolation

1. **Scientific background**

 Loneliness is increasingly recognized as a serious threat to mental health 1,2. Due to the COVID-19 pandemic, social isolation has emerged as an important risk factor related to the onset of several psychiatric and emotional disorders, with the associated quarantine having led to increased domestic violence, fears of job loss, decreased physical activity, altered sleep, and higher levels of anxiety [ Reviewed in 3]. **However, current research has primarily focused on adults, failing to examine the outsized impact of social isolation and loneliness on children and adolescents, whose reactions to COVID-19-related isolation were severe and are thought to be long-lasting**. Social isolation is one of the most prominent stressors in animal models 4. One widely used rodent model of social isolation is the post-weaning isolation approach, which involves separating animals during a very early stage of development (postnatal day [PND] 20-28) without handling, and rearing them in isolation for several weeks or months5. The social isolation of juveniles for 2 weeks from PND 21-35 has been linked to decreased sociability in adulthood and hypomyelination in the deep layers of the medial prefrontal cortex (mPFC), a critical node for social behaviors6,7. Re-socialization of these isolated mice failed to rescue their impaired sociability or mPFC hypomyelination6 (but see 7). Similarly, post-weaning isolation exacerbated inhibitory synaptic activity and decreased intrinsic excitability in pyramidal cells of the mPFC in adult mice. Efforts to restore the sociability of the isolated animals via regrouping, chemogenetic, or optogenetic manipulations failed to achieve more than transient reversal effects8.

 The post-weaning period is a critical developmental window for the maturation of the mPFC, which is required for lifelong emotional and social memory9. This suggests that environmental factors like exposure to adverse stressors in the juvenile stage y can affect the developmental trajectory not only of the mPFC but also of its projections to other parts of the brain. Developing approaches to reverse the deficits caused by social isolation and loneliness in children and adolescents is thus critical, as these deficits develop during a period that sets the stage for lifelong mental health10,11.

Several studies support the hypothesis that an “unhealthy” but palatable diet can mitigate the impact of stress exposure. Indeed, elevated consumption of calorie-rich palatable foods has been shown to engage certain coping mechanisms, thereby reducing stress in humans and rodents12–14. Consistently, long-term high-fat diet (HFD) consumption can selectively protect against some of the behavioral sequelae of chronic unpredictable social stressors15, while also protecting offspring from the consequences of maternal separation stress16. These prior studies have mainly focused on addressing the long-term consequences of social isolation or exposure to HFD after animals reach adulthood. In comparison, very few studies have addressed the effects of these manipulations in young animals. In our published work, we focused on addressing the immediate effects of exposure to either acute stress or acute HFD intake, revealing distinct effects in juvenile and adult animals that are brain region-dependent17–22. In preliminary experiments, we applied social isolation stress, HFD, or both to **juvenile male rats** for one week starting from PND21. Social isolation or HFD alone impaired short-term and long-term social recognition memory as respectively tested with habituation/dishabituation and social recognition memory paradigms (Figures 1-3), while also impairing mPFC synaptic plasticity as measured based on NMDA-dependent long-term potentiation (LTP), reaffirming our previous reports regarding the effects of HFD intake18,23–25. Interestingly, when we presented HFD during isolation, social recognition memory (SRM) and LTP deficits were rescued (Figures 1-4). Notably, this behavioral and electrophysiological rescue persisted **one month later**, whereas previously socially isolated animals fed a control diet still exhibited SRM and LTP deficits (Figure 4). In **adult animals**, HFD alone did not affect SRM23,26 and had mixed effects on LTP, with HFD enhancing hippocampal CA1 LTP21,27 while impairing LTP in the mPFC46, thus revealing brain region-dependent changes distinct from those in juveniles.

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**Figure 1: The acute effects of HFD intake and social isolation for one week on social recognition memory** **in juvenile animals.**

**A:** Schematic overview of the social recognition memory test. Animals were habituated to the arena, and on the next day they were exposed for 1 h to an object or another animal in corrals. After 24 h they are exposed to both the familiar animal and a novel one. For details, see 23,28.

**B****:** Animals at PND21 were weaned and divided into 4 groups: control diet (CD) + group housing (Social-CD), CD + isolation (Isolation-CD), HFD + group housing (Social-HFD), and HFD + isolation (Isolation-HFD). After 7 days of these treatments, animals were subjected to a social recognition memory test. The Isolation-CD and Isolation-HFD did not differ from each other, but did differ significantly from the other groups [P<0.001]. The Social-CD and Isolation-HFD groups showed intact social recognition memory.

**Figure 2: The acute effects of one week of HFD intake and social isolation on SRM measured through habituation and dishabituation in juvenile animals.** On PND21, animals were weaned and separated into 4 groups as in Figure 1. Animals were repeatedly presented with the same conspecific for 4 trials (F1 x 4) separated by 10 min intervals. On the fifth trial, animals were presented with a novel conspecific. For details, see 23,28. While gradual reductions in exploration of the familiar conspecific over repeated trials were observed in the Social-CD and Isolation-HFD groups, the Isolation-CD and the Social-HFD groups maintained enhanced exploration. Upon exposure to a novel conspecific, animals in the Social-CD and Isolated-HFD groups exhibited the recovery of exploration (\*P<0.001).

**Figure 3: The acute effects of one week of HFD intake and social isolation on LTP in the mPFC.** (A) LTP in the indicated groups. (B) Average LTP. Isolation-HFD animals exhibited intact LTP similar to Social-CD animals, while Social-HFD and Isolation-CD exhibited impaired LTP.



**Figure 4:** **The long-term effects of one week of HFD intake and social isolation during the juvenile stage on SRM and LTP tested during adulthood.** On PND21, animals were weaned and divided into 4 groups as in Figure 1. After the 7-day treatment period, they were returned to group housing and CD conditions. Testing was performed on PND60. (A) Only the Isolation-CD group exhibited impaired SRM at this time point, whereas all the other groups exhibited intact memory. \*P<0.01. (B) LTP was intact in Isolation-HFD animals, much as in the Social-CD animals. Interestingly, the Isolation-CD group exhibited impaired LTP (100 ±3.6%) and differed significantly from the Social-HFD group, which showed attenuated levels of potentiation (117.9±2.4%; $ for difference from the Isolation-CD). This suggests that the long-term effect of post-weaning isolation is more severe than the effects of HFD intake.

Social isolation in adults resulted in the impairment of both SRM28 and mPFC-LTP (Figure 5), suggesting that social isolation, unlike HFD, is detrimental in adulthood. Exposure to HFD in isolated adult animals rescued SRM impairment, but only partially reversed mPFC LTP deficits, suggesting that the protective benefits of this combination of stimuli are more potent in juveniles.

**Figure 5: The acute effects of one week of HFD intake and social isolation on SRM and prefrontal LTP in adult animals.** On PND50, animals were divided into the same four groups detailed in Figure 1. After the 7-day diet and housing treatment period was complete, animals were subjected to SRM (A) or LTP (B) testing. (A) Only the SRM of animals in the Isolation-CD group differed significantly from that of animals in the other groups (\*P<0.05). SRM was intact in the Isolation-HFD group. (B) The Isolation-HFD group exhibited LTP that was attenuated compared to the Social-CD group (\*P<0.05), although LTP in this group still differed significantly from the Isolation-CD and Social-HFD groups (& P<0.05), suggesting that HFD does not completely rescue LTP in adults exposed to social isolation.

These findings are consistent with a previous study demonstrating that chronic exposure to HFD after isolation improved isolation-induced deficits in object recognition memory and decreased brain-derived neurotrophic factor (BDNF) levels in mPFC29. Mice fed either high-fat or high-sucrose diets also reportedly exhibit increased rearing behavior in an elevated zero maze 30 days post-exposure to stress and diet conditions, suggesting that even short-term dietary changes can contribute to long-term changes in risk assessment30. These reports suggest that consuming a palatable diet (high in fat or sugar) under stress conditions can limit the consequent stress response. We have recently reported reductions in BDNF levels in the medial amygdala, another important node of social behavior, and impairments in SRM following social isolation for one week in adult animals, while both effects were reversed by the regrouping of the isolated animals 28. Similarly, HFD intake for 72 h in adult animals elevated hippocampal BDNF levels and improved spatial memory27. Together, these studies support our hypothesis that HFD and/or isolation can affect the reprogramming of neural networks in the mPFC and potentially the amygdalar and hippocampal circuits, thereby resulting in long-term consequences.

 With respect to the molecular underpinning of the effects of social isolation on brain circuitry, social isolation in rodents has been shown to affect the expression of non-coding RNAs such as microRNAs (miRNAs) 31–33. miRNAs are a class of ~22 nucleotide short non-coding RNAs that play key roles in fundamental cellular processes, including how cells respond to various stressors34–37. In the brain, miRNAs impact cellular and subcellular functions and modify cognitive performance38,39, including in the context of HFD-induced deficits40. One recent study addressed the effects of social isolation in adult and aged mice and identified relevant changes in miR-140-5p and miR-181a-5p levels in the mPFC and found that these alterations were reversible, returning to basal levels following social enrichment 41. Moreover, the prolonged postnatal isolation of rats resulted in differential miRNA profiles in brain regions involved in anxiety 32. For example, some miRNAs were differentially regulated in both male and female socially isolated rodents, with the majority being downregulated (reviewed in 3). Other studies have attributed the contribution of altered miRNA profiles in the PFC to PFC-dependent tasks38,42. Specifically, miRNA-135a and miRNA-16 were noted as being important contributors to the mechanisms by which stressful early-life experiences increase post-traumatic disorder (PTSD) vulnerability43. Thus, it is likely that specific miRNAs and their target genes are key mediators underlying the deficits caused by HFD or isolation and the rescue of these deficits achieved by HFD intake during social isolation.

 Given the above evidence, ***we hypothesize that HFD intake under conditions of social isolation causes significant cellular and molecular reprogramming that can persistently affect neuronal plasticity and social memory***, with this reprogramming being both age- and brain region-dependent.

1. **Objectives and significance of the research**

Our preliminary data suggest that when HFD or social isolation are presented separately, they induce behavioral and plasticity deficits in the mPFC. However, when presented together, they are able to negate these effects. The resultant protective phenotypes are robust and long-lasting. This unexpected and novel finding is the focus of the present application**.** We thus aim to explore the in-depth behavioral (readout), cellular (circuit), and molecular (mechanism) changes in a specific social memory-related neural circuit comprised of the mPFC, hippocampus, and amygdala in order to reveal the signatures associated with HFD and isolation, with the ultimate goal of understanding how they interact to mediate the reprogramming that occurs at each of these nodes of the circuit.

Stress and HFD result in different effects in the hippocampus and the PFC as compared to the amygdala in an age-dependent manner17,44–47. Social and anxiety disorders are more common in women than in men, yet most animal model studies focus on adult males. As such, the detailed comparisons proposed herein will allow us to examine whether these modifications are differently regulated in the three target brain structures as a function of age and/or sex.

As miRNAs have the potential to influence the expression of multiple mRNA targets via complex miRNA-mRNA interactions, we will seek to identify these targets with the ultimate goal of enhancing or inhibiting relevant miRNAs (using agomirs and antagomirs, respectively), to establish a causal link.

To address our experimental objectives, **we propose the following specific aims:**

**Aim 1:** Profile age- and sex-dependent differences in behavioral changes in response to social isolation and/or HFD intake.

**Aim 2:** Profile age- and sex-dependent activation and electrophysiological signatures in the mPFC-amygdala-CA1 network under conditions of social isolation and/or HFD intake.

**Aim 3**: Explore transcriptomic and proteomic changes to identify and establish the causal roles of miRNA-regulated genes and pathways underlying the phenotypic effects of social isolation and/or HFD intake.

To the best of our knowledge, this proposal is the first to attempt to unravel the molecular effects of social isolation, HFD intake, and the combination thereof at the molecular and circuit levels while assessing two developmental phases (juvenile and adult periods) in both males and females.

**Significance:**

Social isolation, especially during childhood, is detrimental to adult brain function and behavior across mammalian species6,48. While the majority of research performed to date has focused on extended periods of isolation initiated in young mammals followed by the monitoring of resultant effects in adults, here we propose a different approach that uses a short period of acute isolation whereafter we assess the immediate impact of these conditions in juvenile animals as well as their long-term effects. While a growing body of preliminary data pertaining to overweight and obesity rates among children during the COVID-19 pandemic are becoming available, there remains a pressing need to better understand the potential impact of the pandemic on children's health. School closures, strained household finances, increased screen time, and the marketing of fast foods have increased exposure for many children during the pandemic to environmental drivers of weight gain49. Understanding the effects of unhealthy diets on social and cognitive behaviors is thus especially timely in light of these recent increases in social distancing. It is important to note that our acute exposure to HFD was not associated with any metabolic disturbances or weight gain23–26, suggesting that palatable food intake can have context-specific benefits without the negative effects of obesity50. While our HFD contains carbohydrates and is not ketogenic, a growing number of studies have shown that ketogenic diets can improve autistic behaviors characterized by social deficits51, confirming the relevance of behavior-focused dietary research. While the application of a HFD to combat social disturbances in children may be controversial, our proposal is based on a strong foundation of published and preliminary data and is in line with the literature. These findings may thus open an avenue for future characterization of the micronutrients that can overcome social disturbances in children without any associated harmful diet-related effects. This may, in turn, enable the professional design of personalized dietary regimens that can buffer the detrimental effects of social isolation.

1. **Research Plan**

**Aim 1: Profile age- and sex-dependent differences in behavioral changes in response to social isolation and/or HFD intake.**

**Rationale:** Our preliminary findings show that HFD intake was sufficient to rescue SRM deficits in mice exposed to social isolation, with these beneficial effects persisting one month later, whereas social isolation and CD intake was associated with deficits that were still evident at this time point. In contrast to these juvenile animals, only social isolation but not HFD intake resulted in deficits in SRM in adults28. Exposure to HFD in isolated adult animals completely rescued those social memory impairments. Our preliminary and published data focused on the effects of HFD or social isolation were restricted to males 21,23,25,28, and that the effects of these manipulations on juvenile and adult females have yet to be explored. Thus, here in Aim 1 we propose to address:

1. The short-term and long-term age- and sex-dependent effects of HFD, social isolation, and the combination of the two.
2. Whether social isolation induces only social memory deficits or more generalized cognitive and emotional impairments, and whether these impairments are also rescued by HFD intake.

**Aim 1a: Conduct age- and sex-specific behavioral profiling.**

**Rationale:** Even in 2022, preclinical animal models focused on pathological conditions with a high prevalence among females such as anxiety disorders primarily utilize adult male rodents, with many researchers failing to consider sex as an experimental variable 52. Although males and females may be similar at the behavioral level, they often use different mechanisms to respond to social and emotional challenges and opportunities [reviewed in 53]. Notably, our knowledge regarding the mechanisms or the profiles of pre-pubertal females remains very sparse.

We have recently demonstrated that in the social-induced facilitation of extinction, the oxytocin system in juvenile females functions similarly to that of adult males but strikingly different from that of juvenile males20. In preliminary analyses (Figure 6), we found that the engagement of the mPFC in SRM in juvenile females differs from that in juvenile males, more closely resembling that in adult males.

When examining the role of CA1 in social memory and spatial memory, we found that whereas it similarly mediates SRM in both juvenile males and females, it is differentially engaged in spatial memory. Manipulations of the CA1 of juvenile females did not affect spatial memory, whereas in juvenile males such manipulation had a significant effect. These distinct roles were reflected by the differential activation of the neuronal activation marker c-Fos (Shehadeh and Maroun, *Submitted*). This suggests substantial differences between males and females at the pre-pubertal phase. We thus aim to profile the behavioral phenotypes of juvenile and adult females and to compare them to those of males under the three conditions (Isolation, HFD, both) while testing both immediate and long-term effects.



**Figure 6: The effects of the anisomycin-mediated blockade of protein synthesis on the IL-mPFC.** Effects were analyzed in juvenile males (A), adult males (B), and juvenile females (C). Anisomycin microinjection into the IL-mPFC only blocked long-term SRM in juvenile males [t(15)=3.95, \*P<0.001]

**Aim 1b: Profile the cognitive and emotional effects of social isolation, HFD, and the combination of the two in juvenile and adult males and females.**

Social isolation has detrimental effects on human and animal cognitive functions 54,55, and HFD s similarly associated with impaired congition44,56. In our preliminary research, we only focused on social memory. In this aim, we will also evaluate object location memory (OLM), and emotional behavior (anxiety-like behaviors) to address whether the observed rescue effect is restricted to social memory. As exposure to HFD following isolation resulted in a slight improvement in observed deficits29, we will also assess whether there is a critical window for the combination of both conditions by testing whether 1 week of HFD intake immediately after isolation will yield similar rescue phenotypes.

**Working hypothesis:** Based on our published and preliminary data, we hypothesize that sex is a significant factor with the potential to impact behavioral profiles in this model system. It will be interesting to address whether HFD and isolation will exert similar effects in juvenile and adult females as compared to males. Our prior work suggests that it is plausible that juvenile females may exhibit profiles more similar to those of adult males and will be susceptible to social isolation but not to HFD.

**Research design and methods:** Our experimental approach is outlined in Schematic 1. All experiments and protocols proposed herein will be conducted according to the regulations of the ethical committee at the University of Haifa for animal experimentation and welfare. We have experience with all proposed behavioral protocols proposed22–25,45. We will test the effects of Age (Juveniles, Adults), Sex (Males, Females), Housing (Isolation, Group), and Diet (HFD, CD) at 7 or 30 days after manipulations. For behavioral analyses, animals will be tested after the 7 or 30 day periods. Based on our experience, we will require 7-10 animals per group for these behavioral analyses. We will employ the three R policy to reduce the number of animals utilized. To test the temporal contingencies between social isolation and HFD, animals (juveniles and adults of both sexes) will be presented with HFD after the termination of isolation. We will also test additional groups that remain in social isolation with HFD.

**Expected results and pitfalls for Aim 1**: We expect juvenile and adult animals under the combined influence of isolation and HFD to exhibit the restoration of SRM, OLM, and anxiety markers relative to animals exposed to HFD or isolation alone. We expect to observe differences between males and females, although it is possible that no such sex-dependent phenotypes will be observed. If we find that HFD-fed adult female rats exhibit impaired SRM, this will indicate greater vulnerability to HFD in adult females. As to the contingency analyses in which we vary the temporal presentation of HFD and social isolation, we predict that optimal rescue will be observed when isolation and HFD are presented together (rather than in succession). As my lab has expertise in all the required protocols, we do not expect any to encounter any technical challenges.

**Aim 2:** **Profile age- and sex-dependent activation and electrophysiological signatures in the mPFC-amygdala-CA1 network under conditions of social isolation and/or HFD intake.**

**Rationale:** LTP has been described at synapses throughout the brain and remains one of the most attractive cellular models for learning and memory (reviewed in 57). This classical method has yielded exciting results regarding the differential effects of stress on plasticity in juveniles and adults in our lab and others17,19,22,47,58,59. We have previously shown that in juvenile animals, exposure to HFD resulted in the impairment of LTP in both the PFC and the hippocampal CA1 region23–26. However, in adult animals, we found that whereas HFD is detrimental to prefrontal LTP, it enhances its induction in the CA1 region24,26. Furthermore, only partial rescue of LTP was observed in adult animals in the Isolation-HFD group, with significant differences persisting between these animals and the Social-CD group. Interestingly, exposure to HFD in isolated adult animals completely reversed this SRM impairment, suggesting that the mechanisms mediating SRM and LTP may be distinct. Furthermore, the full HFD-mediated rescue of SRM and LTP by HFD in isolated juvenile animals suggests that this combination is more powerful in juveniles. We also have preliminary data demonstrating the differential recruitment of the mPFC in juvenile males but not in adults following SRM acquisition in animals fed a standard control diet (Yaseen and Maroun, *In Preparation*; Image 1). It will be thus interesting to examine whether under isolation, HFD, or both there will be differences in the recruitment of the mPFC, CA1, and amygdala under conditions of isolation, HFD intake, or the combination of the two and whether these effects are age-dependent and/or sexually dimorphic.

 The experimental system that we propose for this aim thus induces changes in both directions (impairment to rescue), has different effects in adults and juveniles, yields distinct effects in different brain regions, and is robust with enduring long-term effects. In this Aim, we propose to examine (1) neuronal c-Fos activation that will be indicative of activated circuits under different conditions and (2) changes in LTP that represent a model of memory formation in the hippocampus-amygdala and PFC circuit.

**Aim 2a: Define the brain regions and circuits activated following exposure to social isolation, HFD intake, or the combination thereof in juvenile and adult males and females.**

To pursue age- and sex-dependent activities that may contribute to the effects of isolation, HFD, or the combination of the two on SRM, we will profile c-Fos expression patterns under different conditions. We will identify the recruitment of the mPFC, CA1, and amygdala (medial and basolateral nuclei) upon social exposure in groups exposed to different conditions (Age, Sex, Diet, and Housing).

**Aim 2b: Explore the electrophysiological signatures associated with isolation and/or HFD intake.**

Our data regarding the differential effects of HFD on LTP in the CA1 and the prefrontal cortex in the adult animals24,26, as well as the partial rescue of prefrontal LTP by the HFD intake, highlight the need to examine the effects of these manipulations in the CA1 or the basolateral amygdala (BLA). For this purpose, we will use high-frequency-induced LTP to identify deficits and rescue phenotypes in this network under our different experimental conditions in juvenile and adult males and females. Stimulation protocols have been described in prior work from our group and others19,21,26,46,47,60.

**Working hypotheses:** Based on our previous publications17,18,47, we hypothesize that the rescue of memory deficits in Aim 1a will be accompanied by the normalization of LTP changes in the CA1 and the mPFC, but not in the BLA. This rescue may also be reflected by differential activation, as monitored by c-Fos levels, when comparing the HFD and isolation groups. As with SRM, we predict optimal rescue when isolation and HFD are presented together (rather than successively) during the juvenile period.

**Research design and methods:** Our experimental approach for this Aim is depicted in Schematic 1. We have experience with all of the protocols proposed herein, with behavioral testing techniques and LTP analyses being performed as described previously18,19,22–25,45. The c-Fos immunostaining protocols are also described in our published work61,62. We will test Age (Juveniles, Adults), Sex (Males and Females), Housing (Isolation, Group), and Diet (HFD, CD) at 7 or 30 days after these experimental manipulations. Behavioral analyses will be performed after 7 or 30 days, and some animals will be used for electrophysiology analyses or will be euthanized 90 min after the last behavioral manipulation. Naïve animals will also undergo electrophysiology and c-Fos as additional controls For each region, we will quantify the number of c-Fos+ neurons from the left and right hemispheres and the average within each animal. We will use 6-8 animals/group for electrophysiology analyses, and 4-5/group for c-Fos staining.

**Expected results and pitfalls for Aim 2**: We expect to detect differences in juveniles and adults, with more robust changes being evident in juveniles relative to adults. If we find that social isolation also impairs cognitive tasks and that HFD intake under isolation can rescue cognitive deficits, this may indicate that isolation and HFD under isolation may have more generalized effects that are not only restricted to social behavior. If we detect overlap in c-Fos activation after HFD and isolation, this may be indicative of shared neural networks. My lab has expertise in all the required protocols and we thus do not expect to face any technical challenges when completing this work. For the mPFC we will be able to differentiate between the different subregions as we have preliminary data to show that the prelimibic and infralimbic areas are differentially engaged in SRM (Yaseen and Maroun, *In Preparation*).

**Image 1**. (A) Representative schematic overlays of c-Fos expression in the PL and IL. (B) Representative images of c-Fos immunohistochemistry staining in the PL and IL following long-term SRM in adult (Top) and juvenile (Bottom) male rats. These show that following the test phase (24 h after the sample phase), juvenile animals exhibited more activation of the IL and PL than did adult animals.



**Schematic 1**: Experimental procedures for Aims 1 and 2. Animals will be weaned at postnatal day (PND) 21 and divided into are divided into four groups. Two animals will be taken from each litter to avoid litter effects. Animals will be caged either in groups (social) or alone (isolation) and fed either HFD or CD. On day 7 they will undergo behavioral testing, and at the end of testing, they will be returned to their experimental conditions. Other animals will be anesthetized for electrophysiological analyses or taken after testing is complete to analyze c-Fos expression. In different groups of animals, after exposure to social isolation and/or HFD for 7-9 days, animals will be returned to social ground housing conditions and fed CD, with testing (behavior, LTP, cFos) then being performed after one month. Adult animals will undergo similar procedures, but social isolation/HFD will be initiated at PND60. For these experiments, we will test males and females, juveniles and adults.

**Aim 3:** **Explore transcriptomic and proteomic changes to identify and establish the causal roles of miRNA-regulated genes and pathways underlying the phenotypic effects of social isolation and/or HFD intake.**

**Rationale:** Our data show that the combination of both social isolation and HFD reverses the defects caused by either of these factors in isolation. This provides a unique opportunity to reveal the molecular mechanisms underlying these and to understand whether the rescue of these deficits corresponds to a unique molecular signature. Many studies have explored the effects of social isolation, stressors, and HFD intake on miRNA expression profiles in different brain regions, including the mPFC1–9. Our preliminary study conducted in collaboration with Prof. Irit Akirav (Psychology Dept. of the University of Haifa) highlighted the influence of early life stress on the expression of miR-16 (Figure 7), revealing that antagonizing miR-16 reduced its expression in the mPFC. We further demonstrated that early-life stress downregulated the expression of miR-16 and that chronic treatment with URB normalized this effect.

**Figure 7: The expression of miR-16 in the mPFC following the microinfusion of anti-miR-16.**

(A)Rats were microinjected with antagomir-16 (anti-mir, 20 nm; Creative Biiogene) in the right ventricle and were decapitated after 7 weeks. A significant decrease in miR-16 expression was observed in the PFC (n=5 in all groups) (\*P<0.05). (B) The effects of early life stress (ELS) and chronic treatment with URB597 during late adolescence on the expression of miR-16 in adult male rats in the mPFC (\*P<0.05; \*\*\*P<0.001; #P<0.05 vs. NoELS-Vehicle) (n=5-10/group).

Therefore, the involvement of miRNAs in the reprogramming of gene expression following dual exposure to social isolation and HFD is highly plausible. Furthermore, as miRNAs regulate protein expression both directly and indirectly, concurrent proteomic analyses are required to fully understand such reprogramming. Indeed, studies have reported changes in proteomic profiles in different brain regions following social isolation 1,10, as did our study of the medial amygdala following social isolation in adult animals11. Here, we aim to conduct genome-wide analyses of both miRNA and proteomic profiles in order to assess how they are affected by social isolation, HFD, and the combination of the two. By integrating the resultant miRNA and proteomic profiles, we will be able to identify uniquepatterns of miRNA expression and complementary alterations in molecular pathways, allowing us to generate robust hypotheses regarding the molecular mechanisms underlying deficiencies caused by HFD or social isolation and their rescue when the two are presented together. After identifying candidate miRNAs that constitute a key signature in the rescue effect, we will aim to establish a mechanistic link by directly manipulating the expression of these miRNAs via the infusion of antagomir/agomir constructs and the monitoring of behavioral outcomes.

**Aim 3a**: **Explore transcriptomic and proteomic changes to identify putative miRNA-regulated genes and pathways underlying diet- and/or isolation-related phenotypes.**

The rescue of SRM and plasticity by two factors that separately cause deficits strongly suggests that distinct molecular signatures underlie HFD/isolation and the combination of the two.

**Working hypotheses**: We predict that different patterns of miRNA and protein expression will be observed in the brain under the three tested conditions (isolation, diet, and isolation+HFD). In accordance with these assumptions that we will observe differential miRNA expression patterns in different brain regions, a previous study reported that the central amygdala and the hippocampal CA1 exhibited distinct clusters of miRNAs following either acute or chronic stress exposure12. It is also plausible that the three conditions may exhibit different molecular signatures.

**Research design and methods:** In order to identifythe molecular mechanisms involved in the deficits observed following either social isolation or HFD exposure and the rescue thereof in response to the combined treatment, integrated miRNA and proteomic analyses will be performed. Due to the high costs of such analyses, we will focus on the brain region that shows the clearest phenotypes in c-Fos and LTP analyses defined in Aim 2 and that may exhibit differential involvement in juveniles and adults. We will sample both males and females in case sex-dependent patterns are observed. For each group, five animals will be sampled after the termination of the 7-day exposure period. We will extract protein and total RNA, including miRNAs, from the same sample as described previously11, and we will additionally collect samples from the other components of the circuit to enable future studies as appropriate. miRNA-Seq library construction and sequencing will be performed at the Technion Genome Center (Technion, Israel). For miRNA-Seq, Pre-processing, identification, and quantification of miRNA expression will follow the workflow proposed by Yao et al. 13. Briefly, following quality control (adapter sequence removal and read-quality filtration), high-quality reads will be mapped to several small RNA debases (including miRBase, piRBase, and GtRNAdb) as well as to the latest NCBI versions of the *Rattus norvegicus* transcriptome and genome (GenBank assembly mRatBN7.2; accession: GCA\_015227675.2). Novel miRNA discovery will be conducted using the miRDeep2 algorithm14. Additionally, we will assess putative mRNA targets of the identified miRNAs using appropriate databases (*e*.*g*., miRDB 15). Label-free LC-MS/MS analysis and determination of proteomic profiles will be conducted at The Smoler Protein Research Center (Technion, Israel). The database for retrieval of the secondary mass spectrometry data will be the Uniprot rat reference proteome, and label-free Quantitation (LFQ) values will be assigned. The datasets obtained through this approach (miRNA x sample and protein x sample) will then be analyzed for differential expression and functional gene-set enrichment. First, each set will be analyzed individually, following our previous workflow11. Next, the two datasets will be integrated using graph convolutional networks, as implemented in *e*.*g*., MOGONET16 via a supervised multi-omics integrative analysis approach. This strategy will allow for the identification of unique biomarkers (both miRNAs and proteins) specific to groups and/or phenotypes. Dr. Maya Lalzar, head of the University of Haifa Bioinformatics Services Unit, will conduct these Bioinformatics analyses.

**Aim 3b: Establish the causal role of specific miRNA candidates by manipulating their expression and assessing whether they can reverse the social deficits caused by social isolation or HFD.**

The list of miRNAs identified in Aim 3a will be cross-referenced with a list of candidate miRNAs specifically associated with social isolation, HFD, and/or stress in the literature (such as miR-218 or miR-16). After identifying these candidate miRNAs, in this sub-aim, we will explore their mechanistic functions by directly manipulating their expression through the infusion of antagomirs/agomirs into the mPFC, CA1, or BLA based on the region profiled above.

**Working hypothesis**: Based on the predictions from Aim 3a and the literature, we hypothesize that activating specific miRNAs that were differentially upregulated in the Isolation + HFD condition will rescue observed deficits in SRM and LTP under the tested conditions. We further expect that these effects will persist 1 month later and that the effects of these manipulations will not only be region-specific (mPFC vs BLA) but also age-dependent, with juvenile animals showing the most robust effect. It is possible that sex-dependent differences will also be observed.

**Research design:** We will rank different miRNAs based on the available evidence and select 20 for validation by reverse-transcription quantitative PCR (RT-qPCR). For these RT-qPCR-confirmed miRNAs, we will microinfuse appropriate antagomirs/agomirs in the selected brain region. For those miRNAs exhibiting a high degree of rescue efficiency for either social isolation, HFD, or social isolation+HFD we will further examine the expression of predicted protein targets, based on the findings in Aim 3a, via Western blotting. Additionally, for the infusion-confirmed miRNAs, we will examine their expression in the other structures sampled in the previous aim. We have extensive experience performing mircoinfusions in different brain structures in both adults and juveniles, enabling us to effectively complete this aim17–21.

**Validation of miRNA expression results by RT-qPCR**: The expression of target miRNAs will be analyzed under different conditions (social isolation vs. no isolation, juveniles vs. adults, males vs. females, HFD vs. CD, 1 week vs. 1 month) in samples of mPFC, BLA, MeA, and CA1 tissue. Briefly, bilateral samples from the punched area will be harvested, and RNA extraction and cDNA synthesis will be performed as per Zaidan et al., (see support letter). The expression of candidate miRNAs will be assessed via SYBR Green-based RT-qPCR amplification. Fold-change values will be calculated using the ΔΔCt method 22 relative to the housekeeping genes RNU6 and RNU66.

**Western blot analysis:** Western blotting analyses will be performed as per our previous work17,18

**Expected results and pitfalls for Aim 3**: We expect to gain insight into the molecular mechanisms underlying social memory impairment in the selected brain area under conditions of social isolation and HFD intake, identifying miRNA and protein biomarkers associated therewith. Importantly, we will identify the molecular pathways that govern the observed rescue phenotypes in response to the combination of social isolation and HFD that occurs in juveniles but not in adults (see Figure 5, the still attenuated level of LTP in Isolation+HFD adult group compared to CD and compared to juveniles that had full rescue in both SRM and LTP). At the conclusion of this aim, we will propose a list of miRNAs and potential miRNA target genes putatively governing the observed reprogramming under different conditions. While they can be effective, bulk tissue analyses have been criticized in brain research for the masking of effects that may only be evident in a small subset of cells within the tissue. As such, our efforts to identify molecular elements may be somewhat incomplete. Nevertheless, our previous experience indicates that the impact of social isolation on transcriptomic and proteomic profiles is readily accessible through bulk RNA- and protein-based analyses11. Additionally, we may find that co-isolation of total RNA and total protein from the same sample may affect the quality or quantity of the sample. In that case, we will extract these different components separately using the left and right hemispheres (counterbalanced).

When establishing a mechanistic link in Aim 3b, we expect that blocking these miRNAs in the Isolation-HFD condition will impair SRM and LTP. In contrast, mimicking their activity will prevent the deficits caused by social isolation or HFD. As the mPFC and amygdala present opposing effects, we predict that we will observe differential effects of activation and inhibition in these compartments. With respect to the long-term effect of miRNA manipulation, we may address the durability of the effects of these agomirs and antagomirs last. If the changes in the expression of the specific miRNAs following microinjection do not last 1 month, we may consider examining animals at 1 or 2 weeks after the termination. However, in our preliminary results we found that antagonizing miR-16 resulted in its persistent downregulation for 7 weeks. As a control, we may also incorporate additional groups and we will microinfuse into the lateral ventricles to compare the resultant phenotypes with those following intra-region microinfusion.

**Research design and methods:**

**Validation of miRNA expression results by RT-qPCR**: The expression of target miRNAs will be analyzed under different conditions (social isolation vs. no isolation, juveniles vs. adults, males vs. females, HFD vs. CD, 1 week vs. 1 month) in samples of mPFC, BLA, MeA, and CA1 tissue. Briefly, bilateral samples from the punched area will be harvested, and RNA extraction and cDNA synthesis will be performed as per Zaidan et al., (see support letter). The expression of candidate miRNAs will be assessed via SYBR Green-based RT-qPCR amplification. Fold-change values will be calculated using the ΔΔCt method 73 relative to the housekeeping genes RNU6 and RNU66.

**Western blotting:** Western blotting analyses will be performed as per our previous work61,62.

**General Pitfalls and Challenges**:

**Types of diets**: The HFD that we characterize here may not be the type of western diet (mixed fat and sugar) that is increasingly consumed in young children and adolescents. We may consider comparisons with other palatable diets, initially focusing on one diet (HFD) due to the large amount of work required.

**Correlations with behavior**: Our behavioral and physiological assays are likely to affect the brain transcriptome. As such, different animals that are exposed to our experimental manipulations but not to a batter of testing may need to be used for RNA-Seq analyses. This may increase the variability in our results, necessitating the testing of more animals.

**Technical challenges:** My lab has extensive expertise in behavior, pharmacology, electrophysiology, and immunohistochemistry such that we do not anticipate major challenges when completing our first two Aims. Our exciting preliminary data require that we step out of our scientific comfort zone to better explore the mechanisms mediating these effects. While my lab has not published on miRNAs, we have attained in-house technical support for miRNA-focused experiments through the laboratory of Prof Irit Akirav with whom we have joint projects focused on the effects of early life stress on the mPFC (preliminary data #7). We have published data with Prof. Shlomo Wagner on transcriptomics (Lavenda et al., 2022 Molecular Psychiatry) as have worked with Profs. Kobi Rosenblum and Inna Gaisler-Solomon. We also have a support letter from Prof. Haitham Amal of the Hebrew University (attached), and will obtain technical support from the Neurobiology and Psychology Departments (Prof. Kobi Rosenblum, and Prof. Inna Gaisler-Solomon) as well as the Unit of Bioinformatics at Haifa University

**I think to say more about the bioinfomatics analysis. What are the expected outputs? What are you going to decipher? Biological process, signaling pathways, cellular compartments?**

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