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Multisensory Stimulation Reverses Memory Impairment in Adrβ3KO Mice

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Abstract: Norepinephrine plays an important role in modulating memory consolidation and evocation through its beta-adrenergic receptors (Adrβ: β_1, β_2 and β_3), which are expressed in the hippocampus (HC) and amygdala (AMY). Here we hypothesized that multisensory stimulation would reverse the memory impairment caused by the inactivation of the Adrβ_3 with consequent inhibition of sustained glial-mediated inflammation and glutamatergic depuration. To test this, 21- and 86-day-old Adrβ_3KO mice and respective controls underwent to (i) gustative and olfactive stimuli of positive and negative valence associated with (ii) intellectual challenges to reach the food in addition to (iii) objects in hidden places (iv) foraging for 8 weeks followed by (v) analysis of GFAP, Iba-1 and EAAT2 protein expression in the HC and AMY. While this protocol restored the memory impairment when applied to Adrβ_3KO animals immediately after weaning, it had no effect when applied to adult animals. In fact, we observed that aging worsens the memory of Adrβ_3KO mice. Although no significant expression of GFAP and Iba1 were observed in HC of young and old mice, Adrβ3KO increased EAAT2 expression HC of old mice, while MS didn't change EAAT2 in young mice but enhanced it expression in older. Relative to AMY of old mice Adrβ3KO increased GFAP expression compared whit WT mice and MS sustained the GFAP expression and increased the EAAT2 expression compared with WT group. These results suggest that a richer and more diverse environment helps to correct memory impairment when applied right after weaning Adrβ_3KO animals, also show that the changes in GFAP, Iba1 and EAAT2 expression levels in young and old mice indicate a functional significance in the process of learning and memory and that the control of neuroinflammation in limbic areas mediates this response. They also reinforce the idea that disruption of noradrenergic signaling could be involved in the cognitive impairment observed later in life.

Keywords: noradrenaline; cognitive benefits; memory; aging; environmental enrichment

1. Introduction

Norepinephrine (NE) has a well-established role in modulating memory consolidation [1,2] in mammals through the activation of beta-adrenergic receptors (Adrβs) expressed in the hippocampus (HC) and amygdala (AMY) [3,4]. The noradrenergic system is known for strengthen long-term potentiation (LTP) within the dentate gyrus of rats during arousing experiences such as exposure to novelty [5,6]. This also drives neuronal activity in the locus coeruleus (the main point from which

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noradrenergic neurons project throughout the brain), and is partially blocked by inhibition of Adrβs [7,8]. Although the role of Adrβ1 and Adrβ2 is well established, a recent increase in experimental evidence has been bringing light to the role of Adrβ 3 as a key role in mediate memory consolidation in rodents [9,10]. Confirming its relevance, the administration of Adrβ3 agonist reversed the memory impairment of animal models for Alzheimer's disease [11].

There is extensive evidence that multisensory stimulation (MS) profoundly affects animal behavior. Specifically, heightened sensory stimulation and problem-solving opportunities enhance performance on various learning and memory tasks [12–14]. MS encompasses different types of stimuli, such as physical, nutritional, sensorial, cognitive, and social [15]. MS is known to increase neurogenesis [16,17] and has been shown to improve learning and memory consolidation in several animal models [18–22]. Male offspring of hypothyroid rat dams exhibited impaired mood and cognition that persisted during adulthood that were significantly improved after 8 weeks of multisensory stimulation [12]. Anxiety, depression, and impaired cognition in the male offspring of hypothyroid dams were significantly improved after 8 weeks of MS [12]. Music training for approximately 3 years reversed the reduction in the size of the HC in children with congenital hypothyroidism [13]. The activation of astrocytes and microglia can cause cognitive decline and memory impairment (in part or totally), effects that can be identified by upregulation of glial fibrillary acid protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba-1) [14–16]. Reactive astrocytes have been increasingly recognized as key contributor to progression of many neurodegenerative diseases [17]. Deletion of astrocytic EAAT2, the major glutamate transporter in the brain, leads to early deficits in short-term memory and in spatial reference learning and long-term memory [18]. Considering that the inactivation of Adrβ$_3$ induces a significant impairment in short- and long-term memory [10] and that the neuroinflammation controls it [19], we hypothesized that the use of MS could reverse the memory impairment exhibited by the Adrβ$_3$ knock-out (Adrβ$_3$KO) mice. The cognitive impairment exhibited by Adrβ$_3$KO mice at 120 days of life was rescued when an 8-week program of MS was initiated after weaning at 21 days of life. However, when the same 8-week MS program started at 120 days of age, the cognitive impairment persisted. Thus, our study aimed to evaluate the effect of a MS environment on memory consolidation processes of young and adult Adrβ3KO mice.

2. Results

2.1. Ambulatory and Exploratory Activity of Adrβ$_3$KO and WT Mice Exposed to MS Early in Life

The MS exposure did not affect the ambulatory activity in the OF test (Figure 2A) but decreased the time spent in the periphery of the OF in both WT and AdrβKO mice in the 3rd day of observation (Figure 2B). The MS protocol decreased the exploratory behavior only in AdrβKO mice in the 3rd day of observation (Figure 2E). The Bonferroni's comparisons test showed decreased exploratory activity in the ARβ3KO-EE group relative to the ARβ3KO group (p = 0.02).

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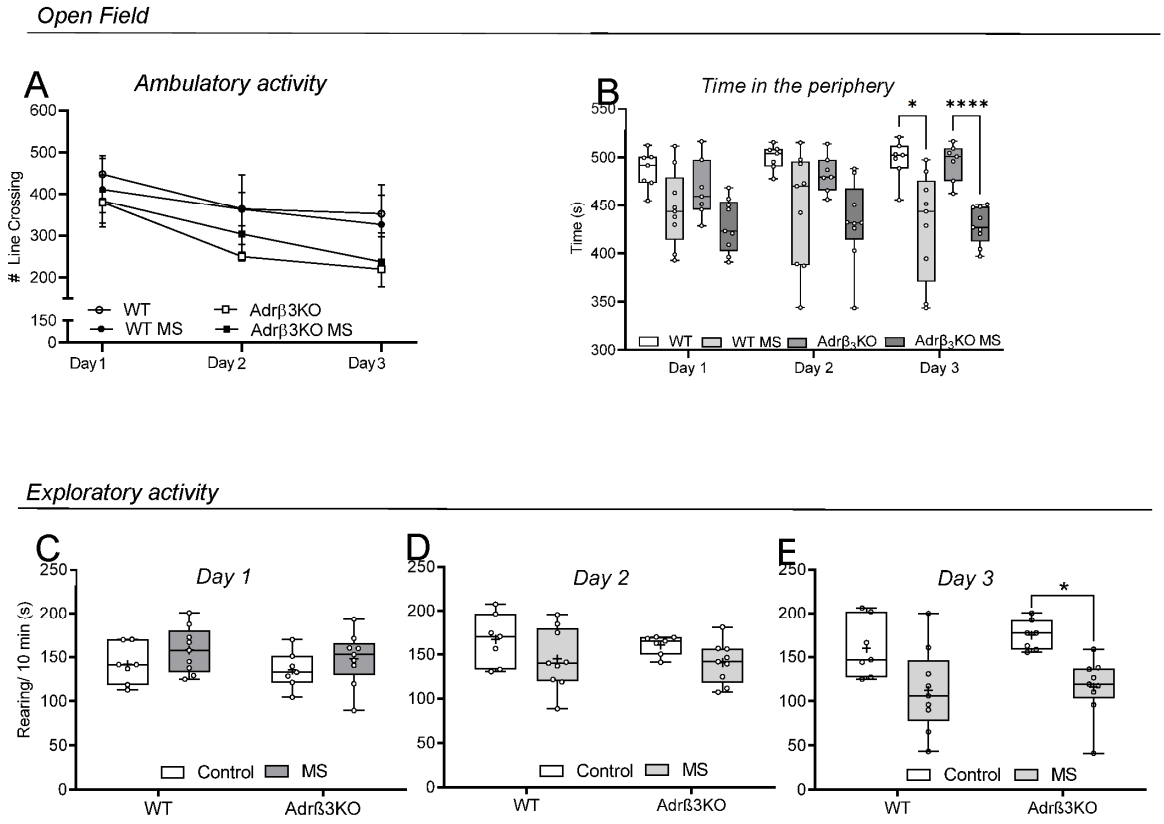

Figure 2. Effect of exposure to EE early in life on locomotor activity and anxiety behavior in young adult Adrβ3KO and WT mice. *Open Field Test*. (**A**) All animals of all groups of young mice exhibited significantly less ambulatory activity along the days of observation (p = 0.025) with no difference among groups; (**B**) Only on day 3 of observation we observed that the MS protocol decreased the time spent in the periphery in the OF of WT group (* p = 0.038) and in the Adrβ3KO (**** p < 0.0001). There was no difference in time spent in the periphery during days 2 and 3 of observations for all groups; (**C**–**E**) There was a decreased in total number of rearings for the Adrβ3KO MS vs. the Adrβ3KO mice only on the 3rd day of observation (p = 0.016), but not on the 1st or 2nd day of observation. There was no difference for WT in all 3 days of observations. All the results were analyzed by 2-way ANOVA with Bonferroni's post-hoc test. Values are expressed as median ±SE (A) or as median (25th percentile–75th percentile) (B-F) (WT n = 7; WT MS n = 9; Adrβ3KO n = 7; Adrβ3KO MS n = 9).

2.2. MS Exposure Early in Life Corrects Cognitive Impairment in Young Adult Adrβ3KO Mice

Cognition was evaluated through the novel object recognition test (NOR) and the valence-based SR. The NOR test uses the preference for novelty exhibited by the rodents, and if they spend more time exploring the novel object it means that they remember the familiar to whom they were exposed before. In our study, the test evaluated short- (3 h) and long-term memory (24 h). SR refers to the ability of animals and humans to discriminate between a familiar and unfamiliar conspecific and is also used to assess memory in rats and mice and does not require the application of additional stimuli to provoke the response. It is used as an index for memory performance [31].

In the NOR test, all groups explored the two identical objects (O1) similarly during the familiarization period (Figure 3A). Three hours after the familiarization period (Figure 3B) the mice were exposed to the O1 and to a new object (O2). The Bonferroni's multiple comparisons tests showed that the absence of Adrβ3 impaired the short memory, but the MS protocol rescued the performance of Adrβ3, with increased time spent with the new object (O2). The WT and the WT MS mice exhibited a preserved short memory (Figure 3B). Twenty-four hours after the familiarization period, The Bonferroni's multiple comparisons test showed that the absence of Adrβ3 affected the long-term memory since the mice spent similar time with the known O1 and with the new O3 (Figure 3C). The

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MS protocol improved the ability of Adrβ3KO mice to remember O1 since they spent significantly more time with O3 (Figure 3C). The WT mice retained the long-term memory with or without the MS protocol.

In the SR test, all groups explored the empty cups similarly during the familiarization period (Figure 3D). When exposed to an empty cup and to a conspecific mouse, all groups preferred to spent time with exploring the cup with conspecific mice showing that the absence of Adrβ3 does not impair the socialization behavior (Figure 3E). In the social discrimination test (Figure 3F) WT, WT MS, Adrβ3KO, and Adrβ3KO MS mice spent significantly more time with the unknown mice than with the known mice (Figure 3F). The difference in the performance of the Adrβ3KO mice in the NOR compared to the SR test is explained by the fact that the SR test uses conspecific animals, and thus, memory formation is strengthened by stimulus valence.

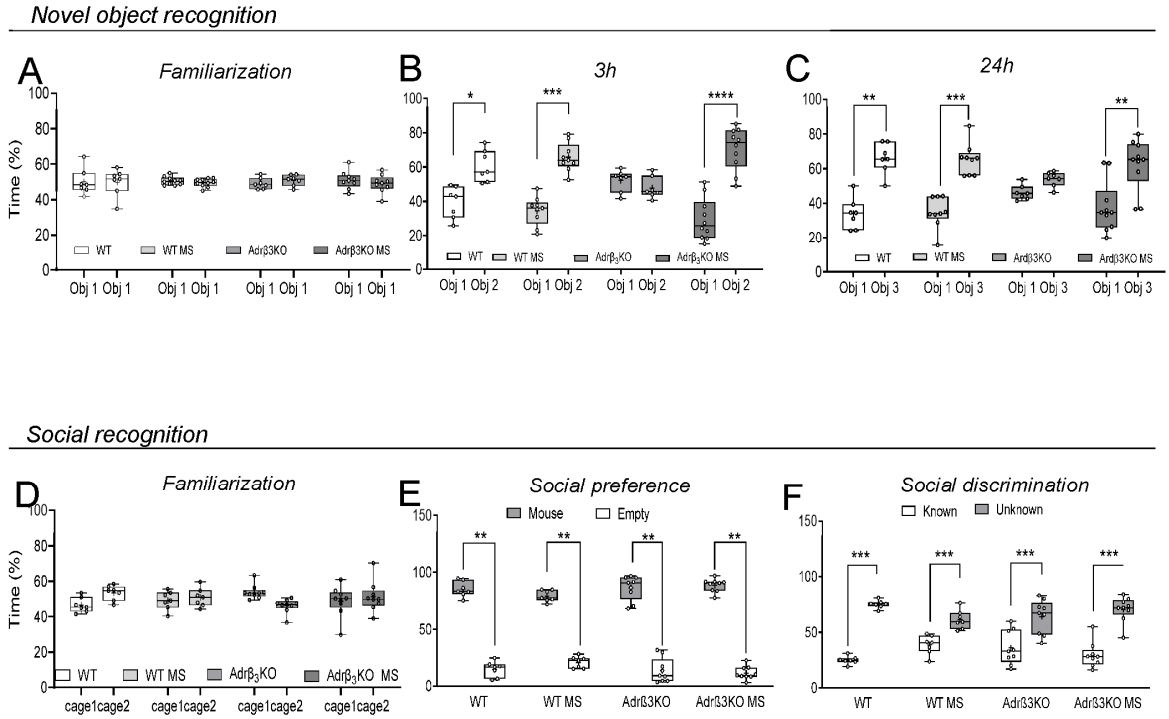

Figure 3. MS exposure early in life corrects cognitive impairment in young adult Adrβ3KO mice: *NOR test in mice without exposure to MS.* (**A**) All groups explored the objects similarly during the familiarization period (**B**) 3 h after object familiarization, WT mice spent significantly more time with a novel object (O2) than a familiar object (O1) (* $p = 0.0252$), while Adrβ3KO mice spent an equal amount of time with both O1 and O2 ($p > 0.9999$). (**C**) 24 h after object familiarization, WT mice spent significantly more time with a novel object (O3) than a familiar object O1 (** $p = 0.0016$), while Adrβ3KO mice spent an equal amount of time with both O1 and O3 ($p > 0.9999$). *NOR test in mice with exposure to MS late in life.* (**A**) All groups explored the objects similarly during the familiarization period. (**B**) 3 h after object familiarization, WT mice (*$p = 0.025$), WT MS mice (*** $p = 0.0001$) and Adrβ3KO mice (**** $p < 0.0001$) exposed to MS early in life spent significantly more time with a novel object (O2) than a familiar object (O1). (**C**) 24 h after object familiarization both WT (*** $p < 0.0006$) and Adrβ3KO (**$p < 0.0041$) mice exposed to MS early in life spent significantly more time with a novel object (O3) than a familiar object (O1). *SR test.* (**D**) All groups explored the empty chambers equally during the familiarization period. (**E**) Both WT and Adrβ3KO mice showed normal preference for social interaction and spent significantly more time in the chamber with a mouse than with an empty cup, regardless of whether they had been exposed to MS (**$p < 0.0001$). (**F**) Both WT and Adrβ3KO mice, with or without MS, showed normal preference for social novelty and spent significantly more time in the chamber with an unknown mouse than in the chamber with the now-familiar mouse (** $p = 0.0013$ and *** $p < 0.0001$). The data weres analyzed by 2-way ANOVA followed by Bonferroni's

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multiple comparison test. Values are expressed as median (25th percentile–75th percentile). (WT n = 7; WT MS n = 9; Adrβ3KO n = 7; Adrβ3KO MS n = 9).

2.3. MS Protocol in Early Life Decreases Glial Cell Activation

To verify if the cognition impairment associated with the absence of Adrβ3 was due to glia activation, we measured the expression of GFAP and Iba-1 in the HC by western blot. As we can see in Figure 4A,B, the younger Adrβ3KO mice exhibits an increase in Iba-1, but not in GFAP expression. The MS protocol exposure early in life decreased GFAP expression in HC of both WT and Adrβ3KO mice, while decreased Iba-1 expression only in HC of Adrβ3KO mice. No alterations were observed in EAAT2 expression (Figure 4C).

2.4. Ambulatory and Exploratory Activity of Adrβ3KO and WT Mice Exposed to MS Late in Life

2-way ANOVA analysis showed that the ambulatory activity of the animals when exposed to the OF test (Figure 4A), was not affected by the MS protocol. The time spent in the periphery of the OF was decreased in day 3 of observation only in the Adrβ3KO MS adult mice group when compared to the Adrβ3KO adult mice (p = 0.015). The MS protocol increased the exploratory activity in the WT adult mice on day 1 (p = 0.003), day 2 (p = 0.02), and day 3 (p = 0.0003) of testing (Figure 4C,D). However, MS decreased the exploratory behavior in the Adrβ3KO mice on day 1 (p = 0.01), day 2 (p = 0.02), and day 3 (p = 0.02) of testing (Figure 4D–F). Notably, control Adrβ3KO adult mice explored significantly more than control WT adult mice also not exposed to EE on day 2 (p = 0.008) and day 3 (p = 0.002).

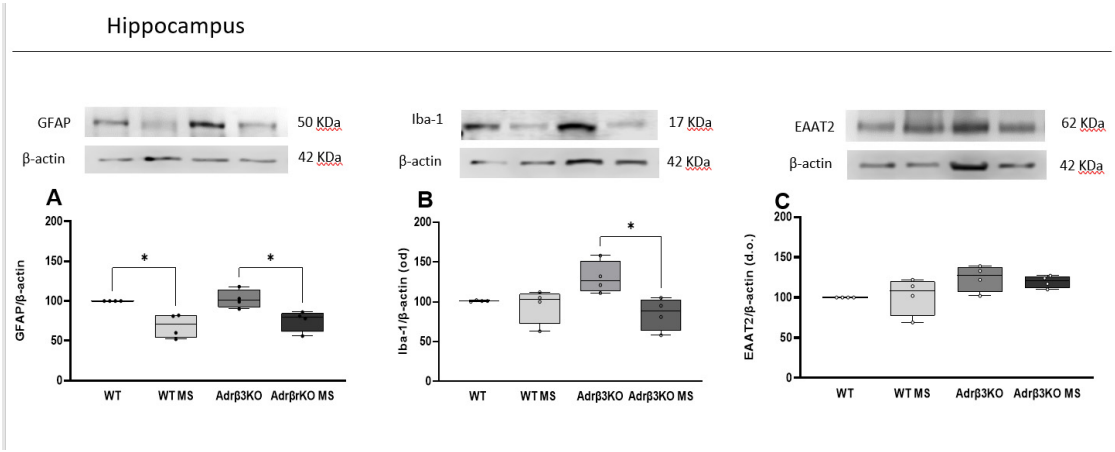

Figure 4. Expression of GFAP, Iba-1 and EAAT2 in hippocampus of young adult Adrβ3KO mice: (**A**) The MS protocol decreased the expression of GFAP in both WT (p = 0.011) and Adrβ3KO mice (p = 0.027); (**B**) MS protocol decreased Iba-1 expression only in Adrβ3KO (p = 0.004); (**C**) EAAT2 expression was not affected by genotype nor MS protocol. The data was analyzed by One-way ANOVA followed by Bonferroni´s pos-hoc test. Values are expressed as median (25th percentile–75th percentile). (WT n = 4; WT MS n = 4; Adrβ3KO n = 4; Adrβ3KO MS n = 4).

2.5. MS Exposure Late in Life Does Not Correct the Worse Cognitive Impairment Observed in Adult Adrβ3KO Mice

In the NOR test, all groups explored the two identical objects (O1) similarly during the familiarization period (Figure 5A). Three hours after the familiarization period (Figure 3B) the mice were exposed to the O1 and to a new object (O2). The Bonferroni's multiple comparisons tests showed that the older Adrβ3KO mice exhibited an impaired short memory, and the MS protocol was unable to rescue or improve this parameter, with similar time spent with the new object (O2). The WT and the WT MS mice exhibited a preserved short memory (Figure 5B). Twenty-four hours after the familiarization period, Bonferroni's multiple comparisons tests showed that the absence of Adrβ3 in

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older mice affected the long-term memory since the mice spent similar time with the known O1 and the new O3 (Figure 5C). Also, the MS protocol did not improve the ability of Adrβ3KO mice to remember O1 since they spent a similar time with O3 (Figure 5C). The WT mice retained long-term memory with or without the MS protocol.

In the SR test, all groups explored the empty cups similarly during the familiarization period (Figure 5D). When exposed to an empty cup and to a conspecific mouse, all groups preferred to spent time with exploring the cup with conspecific mice showing that aging does not impair the socialization behavior regardless the presence of Adrβ 3 (Figure 5E). In the social discrimination test (Figure 3F) older WT and WT MS mice spent significantly more time with the unknown mice than with the known mice (Figure 5F), but Adrβ3KO did not. The exposure to the MS protocol late in life did not rescue this behavior despite the strength of the stimulus (Figure 5F).

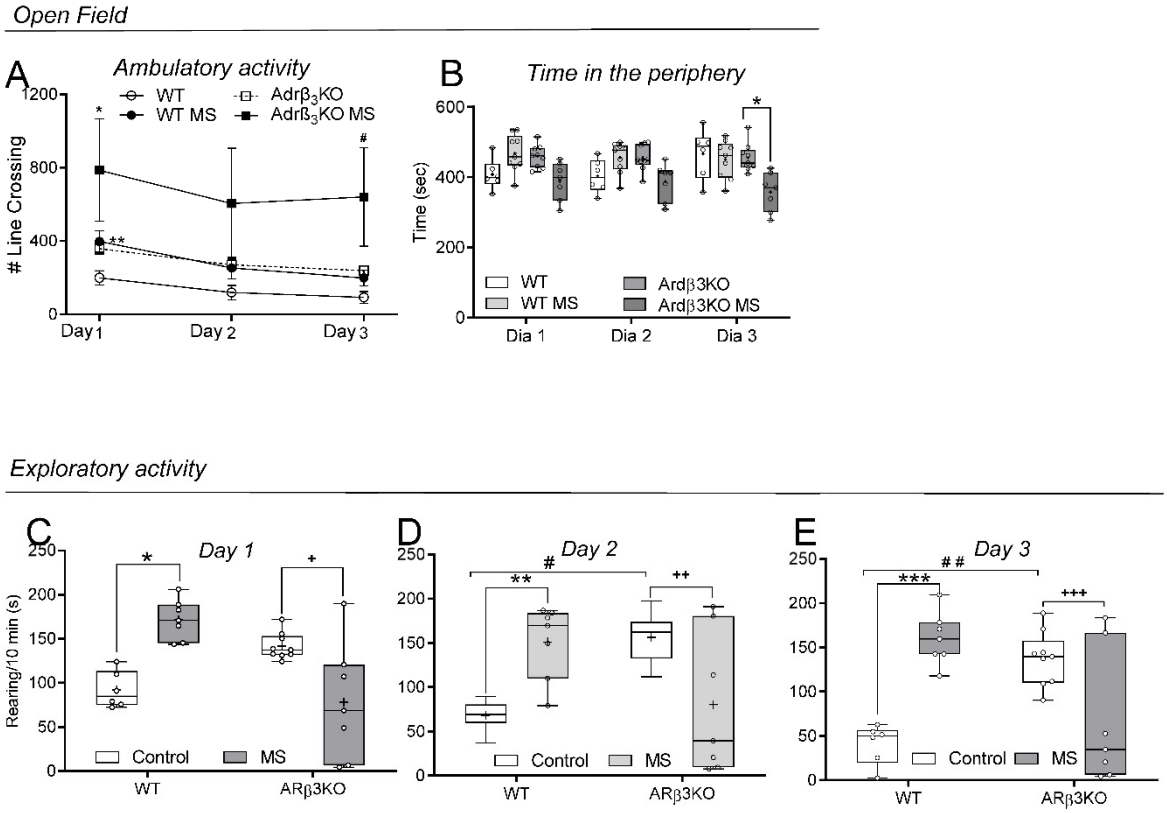

Figure 5. Effect of exposure to MS late in life on locomotor activity and anxiety behavior in young adult Adrβ3KO and WT mice. *Open Field Test*. (**A**) Total number of line crossings of WT and Adrβ3KO mice without exposure to MS and with exposure to MS. On the day 1 of observation, the ambulatory activity of Adrβ3KO was significantly higher when compared to WT mice (p = 0.02). On day 1 of observation the MS protocol increased the ambulatory activity only in the Adrβ3KO MS group when compared to Adrβ3KO (*p = 0.037). On day 3 of observation Adrβ3KO MS exhibited an increase in ambulatory activity when compared to WT MS (p < 0.02); (**B**) On day 3, the Adrβ3KO MS mice spent less time in the periphery than the Adrβ3KO group (** p = 0.015); (**C**,**D**) Total number of rearings were increased in WT MS when compared to WT on day 1 (* p = 0.003), day 2 (** p = 0.02), and day 3 (*** p = 0.0003); Total number of rearing were decreased in Adrβ3KO MS vs. Adrβ3KO adult mice on day 1 ($^{+}$ p=0.01), day 2 ($^{++}$ p = 0.02) and day 3 ($^{+++}$ p = 0.02). The data were analyzed by 2-way ANOVA followed by Bonferroni´s pos-hoc test. Values are expressed as media ± SE (**A**) or as median (25th percentile–75th percentile) (**B**–**F**). (WT n = 6; WT EE n = 7; Adrβ3KO n = 9; Adrβ3KO EE n = 7).

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2.6. MS Protocol Late in Life Does Not Decreases Glial Cell Activation

To verify if the increased decline in cognition observed in older Adrβ3KO mice was accompanied in increased glial activation, we measured the expression of GFAP and Iba-1 in the HC by western blot. The older Adrβ3KO mice did not exhibit alterations in GFAP or Iba-1 expression in the HC (Figure 7A,B). Notably, the MS protocol was not able to decrease the GFAP expression and increased the Iba-1 expression when compared to Adrβ3KO mice (Figure 7A,B). Also, EAAT2 expression was increased in Adrβ3KO mice when compared to WT mice and MS protocol was not able to change the EAAT2 expression in both WT and Adrβ3KO mice (Figure 7C). Considering that Adrβ3KO mice showed an inability to discriminate between a familiar and unknown conspecific (Figure 6F), we performed the analysis of GFAP, Iba-1 and EAAT2 expression in the AMY, a potentially critical site for in emotional processing stimuli, such as an encounter with a conspecific mouse [32,33]. As we can see in Figures 8A, there is an increase in GFAP in the AMY of older Adrβ3KO mice and MS protocol was not able to reduce it. The expression of Iba-1 in the AMY was note affected by genotype or MS protocol (Figure 8B). The EAAT2 expression in the AMY was not affected by genotype but increased in WT MS and Adrβ3KO MS older when compared to WT mice (p = 0.02 and 0.034, respectively, Figure 8C).

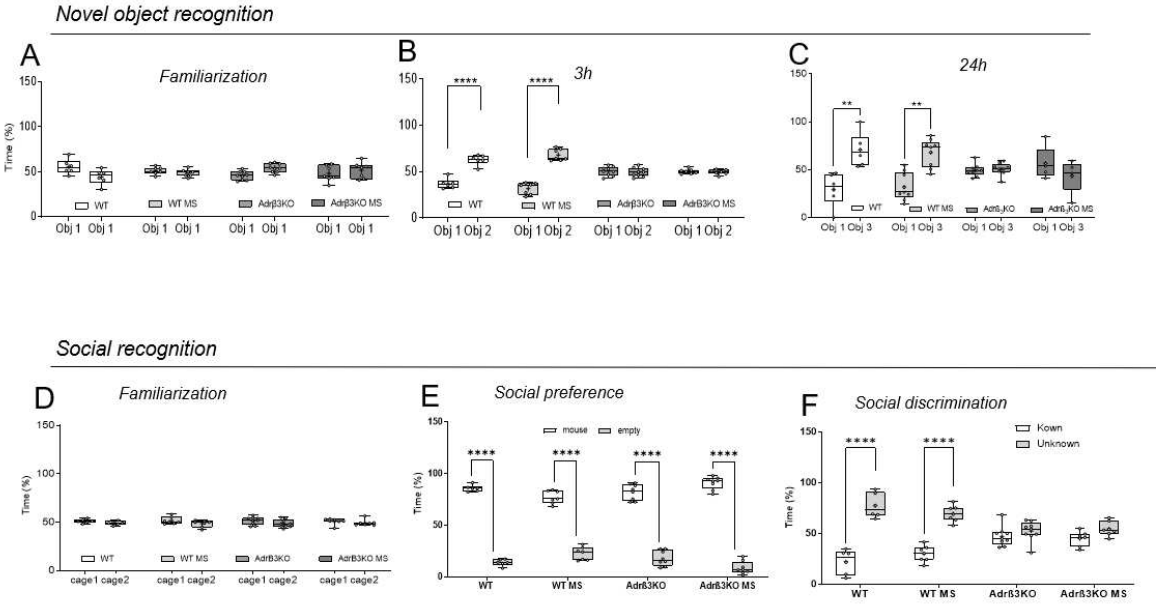

Figure 6. MS exposure late in life does not correct cognitive impairment in adult Adrβ3KO mice: *Novel Object Recognition test*. (**A**) All groups explored the objects similarly during the familiarization period. (**B**) 3 h after object familiarization, WT and WT MS mice spent significantly more time with a novel object (O2) than a familiar object (O1) (**** $p < 0.0001$), while Adrβ3KO and Adrβ3KO MS mice spent an equal amount of time with both O1 and O2 ($p > 0.9999$); (**C**) 24 h after object familiarization, WT and WT MS mice spent significantly more time with a novel object (O3) than a familiar object (O1) (** $p = 0.0040$), while Adrβ3KO and Adrβ3KO MS mice spent an equal amount of time with both O1 and O3 ($p > 0.9999$). *Social Recognition test.* (**D**) All groups explored the empty cambers equally during the familiarization period (**E**) All groups showed normal preference for social interaction and spent significantly more time in the chamber with a mouse than with an empty cup, regardless of exposure to MS (**** $p < 0.0001$). (**F**) Both WT (** $p = 0.004$) and WT MS (** $p = 0.002$) mice showed normal preference for social novelty and spent significantly more time in the chamber with an unknown mouse than in the chamber with the known mouse, while Adrβ3KO mice, with and without MS, spent an equal amount of time with both known and unknown mice ($p > 0.999$). The data was analyzed by 2-way ANOVA followed by Bonferroni´s pos-hoc test. Values are expressed as median (25th percentile–75th percentile). (WT $n = 6$; WT MS $n = 7$; Adrβ3KO $n = 9$; Adrβ3KO MS $n = 7$).

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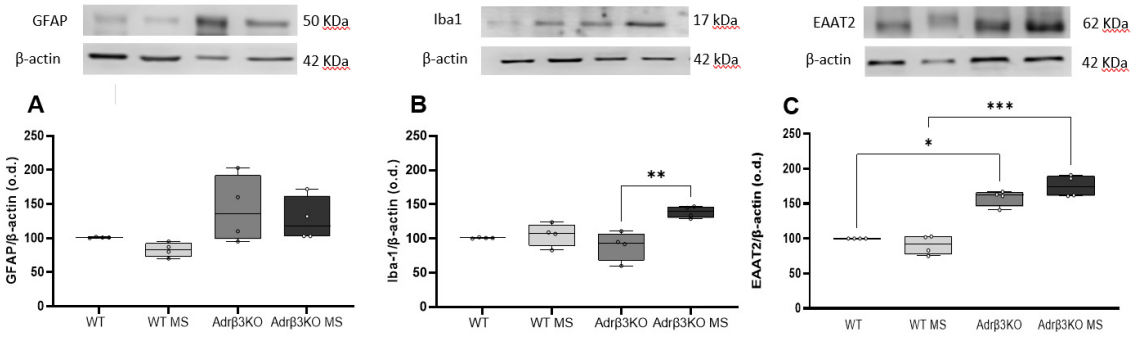

Figure 7. Expression of GFAP, Iba-1 and EAAT2 in hippocampus of older Adrβ3KO mice: (**A**) The expression of GFAP was not affected in older mice regardless the genotype or the MS protocol; (**B**) Iba-1 expression was increased in Adrβ3KO MS when compared to Adrβ3KO mice (p = 0.002); (**C**) EAAT2 expression was increased in Adrβ3KO when compared to WT mice (p = 0.013) and in Adrβ3KO MS when compared to WT MS (p = 0.002). The MS protocol did not affect the expression of EAAT2 regardless the genotype. The data was analyzed by One-way ANOVA followed by Bonferroni´s pos-hoc test. Values are expressed as median (25th percentile–75th percentile). (WT n = 4; WT MS n = 4; Adrβ3KO n = 4; Adrβ3KO MS n = 4).

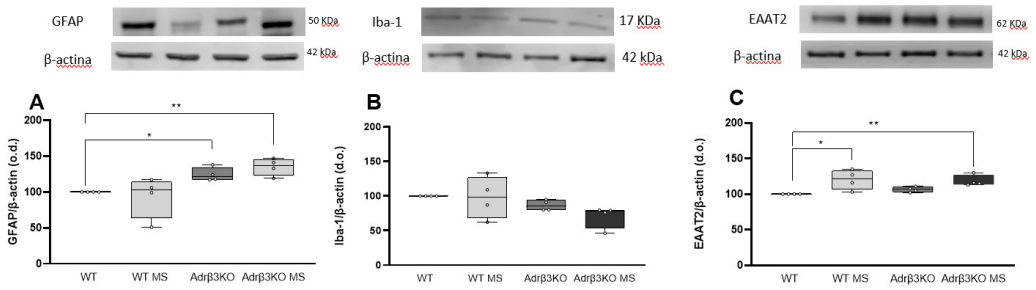

Figure 8. Expression of GFAP, Iba-1 and EAAT2 in amygdala of older Adrβ3KO mice: (**A**) The expression of GFAP was increased in Adrβ3KO when compared to WT (p = 0.045) and in Adrβ3KO MS when compared to WT mice (p = 0.031). The MS protocol did not affect the expression of GFAP regardless the genotype; (**B**) Iba-1 expression was not affected by MS protocol or by genotype; (**C**) EAAT2 expression was increased in WT MS when compared to WT (p = 0.02) and was increased in Adrβ3KO MS when compared to WT MS (p = 0.034) but was not affected by genotype. The data was analyzed by One-way ANOVA followed by Bonferroni´s pos-hoc test. Values are expressed as median (25th percentile–75th percentile). (WT n = 4; WT MS n = 4; Adrβ3KO n = 4; Adrβ3KO MS n = 4).

3. Discussion

The present study revealed that cognitive impairment in younger Adrβ3KO mice is reversed by the MS protocol when it is initiated precociously in life (21 days of age). We also observed that the cognitive impairment worsens with aging in Adrβ3KO mice, and this deficit was not improved by the MS protocol initiated late in life (120 days of age). Interestingly, glial cells and glutamate transporter expression have not been shown to associate with the cognitive impairment seen in younger Adrβ3KO mice, but MS protocol could decrease GFAP and Iba1 expression on HC which might have contributed to revert Adrβ3KO-related cognitive decline. In the HC of aged mice, Adrβ3KO also has not been shown to alter glial cell expression. After the MS protocol older mice exhibited a higher expression of EAAT2, although studies have demonstrated that increased EAAT2

expression may exert beneficial effects on cognitive function, this was not enough to promote cognitive recovery in old mice.

Present results confirm our previous observation that Adrβ3KO mice exhibit a moderate cognitive impairment [10]. Remarkably, this phenotype was entirely reversed by an 8-week MS protocol when it was initiated in very young mice. We know that MS increases hippocampal neuroplasticity [34,35] and neurogenesis [36,37], but we did not address this process in the present study. It is also known that MS decreases neuroinflammation [38,39], and our data gives support to this important role of MS. We showed that there is a decrease in the expression of GFAP, a marker for astrocyte activation, in the HC of Adrβ3KO and WT mice after the 8-week MS protocol. Iba-1, a marker for microglia activation, was also reduced in Adrβ3KO exposed to the MS protocol Mounting evidences indicates a close relationship between glial cells in both cognitive impairments [40,41], and in the pathogenesis of neurodegenerative disorder, such as Alzheimer's disease [42,43]. MS also been showing beneficial effects against inflammation by down-regulating the expression of GFAP and Iba-1 in the HC of Adrβ3KO and WT mice leading to reduced glial cells activation and cytokine-mediated inflammation, and this may contribute to ameliorate cognitive functions and memory [44,45]. NE modulates the activity of microglia and astrocytes [46,47] decreasing the inflammatory markers by binding to β-adrenergic receptors [47,48]. The role of beta-adrenergic receptors in glial cells has been under investigation since late 1990, It has been shown that Adrβ2 present in glial cells modulates astrocytes phenotype and phagocytic activity [49,50], while also modulating the activation of classical activated microglia [51]. However, to the best of our knowledge, this is the first report of glial cell modulation by Adrβ3 manipulation, which may shed light into the importance of this pivotal receptors, not only for neuronal activation, but also glial neuroplasticity.

Older mice exhibited the worst cognition. Younger mice Adrβ3KO can discriminate familiar co-specific and spend more time with the novelty object. Noradrenergic modulation of the AMY is very important for forming emotional memory and interaction with an unknown mouse which involves emotional valence. 6–7-month-old Adrβ3KO mice spent similar time with both familiar and unknown co-specific mouse, showing that they did not remember the familiar mouse to whom they were exposed earlier. Also, the MS protocol initiated later in life did not improve the cognitive deficit exhibited by older Adrβ3KO mice. We could not find alterations in astrocytes or microglia expression in the HC of older Adrβ3KO mice when compared to WT of the same age. A possible explanation is due to the glial modification in the HC of older rodents. Since older animals by itself have an important astrocytic modulation that has been shown to be more evident that the one observed in AD pathology [52] it is rationale to assume that in older Adrβ3KO mice this can be also the case in the HC. However, even though we could not find any differences regarding GFAP expression in the HC, we did find that Adrβ3KO animals have an increase of EAAT2 expression in this nucleus. EAAT2 is the main transporter responsible for the reuptake of glutamate by astrocytes in the synaptic cleft [53]. Glutamate is the major excitatory neurotransmitter in the brain [53], but extracellular excess of glutamate increases the production of reactive oxygen/nitrogen species, which induce oxidative stress leading to neuronal death [54]. EAAT2 regulates and buffers the amount of synaptic glutamate preventing neuronal damage due the glutamate excitotoxicity [55]. Even though the role of EAAT2 is well recognize, its response to different types of stress is under investigation due to its complexity. In this sense, it has been shown that while stroke inhibit the expression of EAAT2, Adrβ -blocker attenuated this inhibition [56]), suggesting that adrenergic receptors have a role in the expression of EAAT2, corroborating with our results. Interestingly, MS was unable to modify the expression of EAAT2 in the HC of older mice.

Regarding the AMY, we did find an increase of GFAP expression in the AMY of Adrβ3KO when compared to WT mice. Also, the MS protocol initiated later in life did not decrease the GFAP and the Iba-1 expression as it did in younger mice. Despite this, changes in the EAAT2 expression in the AMY of Adrβ3KO when compared to WT mice were evident in MS group. The majority of glutamate uptake is through EAAT2 [53], and interestingly lower EAAT2 expression or activity was reported in multiple neurological disorders such as Amyotrophic lateral sclerosis [57], Alzheimer's disease [58] and schizophrenia [59]. Given the evidences of EAAT2 lower expression has an implication among

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brain diseases; we could hypothesize that MS protects against EAAT2 dysregulation, which may play a role in normal cognition [60,61]. Although this hypothesis is only speculative and additional studies are needed to better understand the mechanisms by which EAAT2 expression or activity could alter cognitive functions.

Although we observed an increase in GFAP expression in the AMY of older Adrβ3KO mice, a marker of the astrocyte activation, our data do not support the Adrβ3 as an important adrenergic receptor mediating the anti-inflammatory effects of NE. Nevertheless, Adrβ3 may have a pivotal role in astrocytic modulation, possibly controlling different roles of astrocytes rather than inflammation, such as synaptic pruning and glutamatergic depuration. Further studies are needed to clarify the role of this receptors in the functions of astrocytes. However, our data does show that this receptor is key to the cognition response in young and older mice.

A complex and dynamic MS protocol that exposed the animals to different kinds of stimuli, such as gustative and olfactive stimuli of positive and negative valence, intellectual challenges to reach food, the use of hidden objects, and the presentation of food in ways that promoted foraging, rescued the memory deficit of young Adrβ3KO mice when applied immediately after weaning and seem to do this decreasing neuroinflammation.

The constant exposure of animals to novelty through the MS protocol used in the present study involves some level of stress to the animals. In addition, the animals were exposed to stimuli with negative valence, such as bedding with the smell of rats. Our results suggest that a moderate level of stress experienced early in life could be beneficial for cognition.

The observation that aging worsens the memory of Adrβ3KO mice is notable despite the AMY activation caused by valence of the stimulus. It has been shown that locus coeruleus (LC) degeneration is a common neuropathological feature of neurogenerative diseases such as Alzheimer's disease [62,63]. In fact, early degeneration of the LC could trigger or be involved in the progression of neurogenerative diseases [64–67]. The fact that Adrβ3KO inactivation leads to a greater loss in cognition with aging highlights the role of the noradrenergic signaling pathway in the course of dementia.

In healthy rodents, LC projections to different brain regions begin to decline by 7–15 months of age [68,69]. Other studies with rodents and primates have found a correlation between memory loss and the progressive appearance of lesions, and consequent cell loss in the HC and entorhinal cortex, with age [70,71]. Advancing age leads to a loss of 10 to 20% of brain mass when compared to a young brain. This can lead to variations in cell loss in different brain regions and, consequently, more serious losses in certain regions than in others [72]. The Adrβ3 inactivation, combined with the functional changes typical of advancing age, can aggravate damage to memory formation processes, possibly explaining the worsening in memory observed in the adult Adrβ3KO mice.

The MS protocol used in the present study did not affect locomotor capacity when applied to young animals regardless of the genotype but increased ambulatory activity in older Adrβ3KO mice. This suggests that the stimulus represented by MS may improve the activity of animals at an older age. The influence of MS on the exploratory behavior of mice has already been evaluated in other studies, but there is, yet no consensus on its influence [73,74].

In conclusion, the results of the present study reinforce the idea that early stimulation of individuals is beneficial for cognition and can prevent or delay early memory impairment caused by defects in neuronal signaling involved in cognition. They also showed that the Adrβ3 has an important role in memory as aging worsens the memory of Adrβ3KO animals. The observed expression of glial cells and glutamate transporter support the idea that changes in glial cells, especially the astrocytic response may be an important component of adrenergic modulation and induced cognitive deficit in Adrβ3 knock-out (Adrβ3KO) mice, but further studies will be required to prove this hypothesis.

4. Materials and Methods

Animals: Adrβ3KO mice (*Mus musculus*) with an FVB background were generated by removing the 306bp genomic fragment containing the sequences encoding the third through the fifth

transmembrane domains of the Adrβ3 and replacing it with a neomycin selection cassette, as described by Susulic et al. [20]. We purchased the animals from Jackson Laboratory (Bar Harbor, ME USA) and established an in-house colony at the Animal Facility at Mackenzie Presbyterian University (Sao Paulo, Brazil). All mice used in this study were genotyped using RT-PCR to confirm their status as homozygous knockout (Adrβ3KO) or wild-type (WT) mice. In total 32 male Adrβ3KO mice and 29 male WT controls from different litters randomized between groups were used in a protocol approved by the Institutional Committee on Animal Research at the Center of Biological Sciences and Health, Mackenzie Presbyterian University (CEUA/UPM Nº156/02/2017). Mice were housed in groups at 26 °C, 55–60% humidity, and a 12-h light/dark cycle with *ad libitum* access to standard food (Nuvilab, Brazil) and water. In the current study, we focused on male mice to reduce the number of confounding factors.

Experimental design: We evaluated the effects of Multisensory Stimulation (MS) at two periods in the lives of animals: (i). immediately after weaning on postnatal day (PND) 21 and lasting until PND 85; and (ii) in adult life, with MS starting on PND 120 and lasting until PND 180.

Effect of early MS on young mice: The animals were transferred immediately after weaning on PND 21 to the cage with two floors and were submitted to the protocol described in Table 1 until PND 85. Behavioral tests then started and were completed on PND 120 (Figure 1A). The animals were divided into the following groups: WT (n = 7); Adrβ3KO mice (n = 7); WT MS (n = 9); and Adrβ3KO MS (n = 9).

Effect of late MS on adult mice: The animals were kept in standard housings until PND 120 when they were then transferred to the cage with two floors and submitted to the protocol described in Table 1 until PND 180 (Figure 1B). Behavioral tests were started on PND 180 and finished on PND 205. The animals were divided into the following groups: WT (n = 6); Adrβ3KO (n = 9); WT MS (n = 7); and Adrβ3KO MS (n = 7).

Table 1. Multisensory Stimulation Protocol.

Weeks	1st Intervention	2nd Intervention
1st	Familiarization with the new environment.	Banana (100 g), apple (50 g), grape (25 g) for 5–6 h
2nd	Exposure to cotton balls of different sizes for 4–5 h	Hiding fruit under the bedding for 4–5 h
3rd	Exposure to ice with and without water for 1 h	Exposure to newspaper sheets for 5–6 h
4th	Exposure to carrots (100 g) in different sizes for 4–5 h	Exposure to plastic balls in a box filled with bedding for 3–4 h
5th	Exposure to trail with seasonings (oregano, lemongrass and chamomile) for 2 h	Exposure to cooked rice (50 g) for 3–4 h
6th	Exposure to two extra burrows made from cardboard.	A banana (100 g) hanging from a thread attached to the roof of the housing for 2–3 h
7th	Exposure to mirrors for 15 min	Exposure to neutral jelly with raisins (8 units) inside for 3 h
8th	Exposure to bedding from a rat housing placed in four different locations in the housing for 1 h	Exposure to bowls containing water with frozen peas, carrots, and corn for 2–3 h

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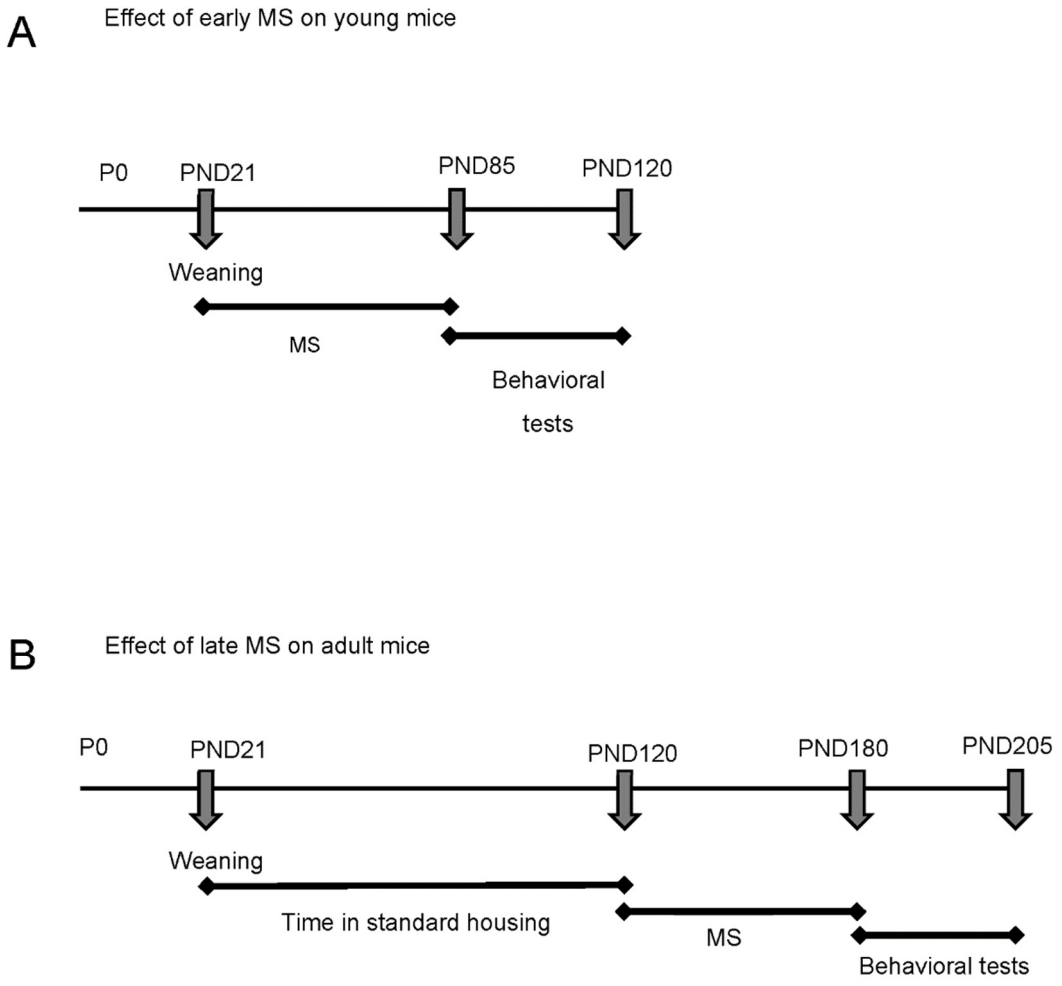

Figure 1. Experimental design. (**A**) Multisensory Stimulation (MS) started immediately after weaning on post-natal day 21 (PND2 1) until PND 85. The behavior assessment started on PND 85 and last until PND 120. During the tests, the animals remained in the stimulatory housing but without the interventions (**B**) MS was initiated in adult life on PND 120 and last until PND 180 when the behavior assessment was performed until PND 205.

Multisensory stimulation protocol: All mice submitted to the MS remained in two-story housings (57 × 31 × 41 cm), lined with wood shavings and with a shelter, water, and chow diet on both floors. The control groups remained in regular housing (a Plexiglas cage measuring 30 × 20 × 13 cm). The MS protocol used was that standardized in our laboratory with some adaptations [21,22] and consisted of two interventions per week for eight weeks in the morning, with sensory, cognitive, and dietary activities, to keep the novelty throughout the whole protocol (Table 1). After eight weeks of MS, the behavioral tests were started. During the behavioral assessment, the animals remained in the two-story housings until the completion of the tests, but without the stimulatory activities.

Behavioral testing: All tests were performed in the morning (7:00–9:00 AM), under dimmed light (15 lux), and recorded by video for later analysis by two different blind observers in the following order for both studies 1 and 2.

Open field test (OF): The open field test was used to evaluate locomotor and exploratory activity [23]. The animals were placed in the center of a circular acrylic arena (diameter = 30 cm) divided into four central zones and eight peripheral zones (Insight Ltd.a, Brazil), in a low-light environment (15 Lux) for 10 min. Locomotion (total number of lines crossed with all four paws) in the central and peripheral zones, and time spent in the periphery were evaluated using the software OpenFLD v 1.0 (OpenFLD v1.0–available at http://blog.sbnec.org.br/2010/07/software-gratuitos-para-analise-do-

labirinto-em-cruzelevado-e-campo-aberto/). Rearing was evaluated manually by two independent blind observers. The test was performed three consecutive times with a 24-h interval [24].

Novel object recognition test (NOR): This test was performed to evaluate short- and long-term memory. It was performed in the OF arena immediately after the OF test to guarantee the habituation of the mice to the arena. The test consists of three stages: familiarization, test (3 h later), and retest (24 h later). In the familiarization stage, the animals were placed in the open field arena for 10 min and were then exposed to two unknown identical objects, object O1 and object O1' for 3 min. Three hours later, the test was performed with the animals being placed in the arena for 3 min and exposed to object O1 and a new object (O2). Twenty-four hours after the familiarization, the animals were placed in the arena for 3 min and exposed to the known object O1 and a new object (O3). At each stage, the time the animal spent exploring the object with their nose was expressed as a recognition index, i.e., the percentage of time spent with each object of the total time spent with both objects [25]. Time spent with each object was evaluated manually by two independent blind observers.

Social recognition test (SR): Social preference and discrimination were evaluated using a non-automated, 3-chambered box with three successive and identical chambers (Stoelting, Dublin). The protocol used is like the one described previously by Moy et al. (2018) [26]. Briefly, in the familiarization period, the mice were allowed to explore the three chambers freely for 10 min starting from the intermediate compartment, with the two other chambers containing empty wire cups. To test social preference, the test mouse was placed in the intermediate compartment, while an unfamiliar mouse was now put in one of the wired cups in a random and balanced manner. The doors were re-opened, and the test mouse was allowed to explore the three chambers for 10 min. The time spent in each of the chambers, the number of entries into each chamber, and the time spent sniffing each wired cup were recorded to measure social preference. In the third phase, social discrimination was evaluated with a new, unknown mouse being placed into the remaining empty wire cup with the test mouse allowed to explore the entire arena for 10 min, having the choice between the first, already-investigated mouse (known) and the novel unfamiliar mouse (unknown). The same measures were taken as for the social preference test [27,28]. Time spent with each cage was evaluated manually by two independent blind observers.

Western blot analysis: Immunoblotting was performed to analyze the expression of GFAP, Iba-1 and EAAT2 proteins with β-actin as an internal loading control according to the procedure described by Towbin et al. [29]. The HC and AMY were homogenized in 200 µL of radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholate, and 1% NP-40] with proteinase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 12,000 g for 15 min at 4 ºC. The supernatant was collected and analysed for protein concentration using the method of Bradford (Thermo Fischer Scientific, Waltham, MA, USA) [30]. The samples were diluted in Laemmli buffer and aliquots containing 30 µg of protein was loaded per lane and separated on 10% SDS-PAGE for GFAP and EAAT2 and on 15% SDS-PAGE for Iba-1 along with unstained protein molecular weight marker (see Supplementary data). The proteins were transferred electrophoretically to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) in a transfer buffer (25 mM Tris–HCl, 192 mM glycine and 20% (*v*/*v*) methanol, pH 8.3). The membranes were blocked for 1 h at room temperature with 5% (*w*/*v*) non-fat dry milk in Tris-buffered saline containing Tween (TBST: 25 mM Tris, pH 8.0, 150 mM NaCl and 0.05% tween 20). Then, washed in TBST, following the membranes were then probed with anti-GFAP, anti-Iba-1 and EAAT2 antibodies diluted 1:500, 1:1000 and 1:2000 respectively in 3% (*w*/*v*) TBST + 1% BSA + 0,1% Sodium Azide overnight at 4 °C. After washing with TBST, membranes were incubated with the corresponding HRP-conjugated secondary antibodies diluted in 2% (*w*/*v*) non-fat dry milk in TBST. The membranes were washed three times with TBST and the detection of proteins was done using a chemioluminescent kit (ECL, Amersham Biosciences, NJ, EUA). The target proteins were detected using a C-DiGit western blot scanner (LI-COR, USA). Densitometric analysis was done using ImageJ software (National Institutes of Health, USA).

Statistical Analysis

Sample size: To determine the sample size, information from a pilot sample with 12 mice allocated in 4 groups (WT, AdrB3KO, WT MS, and AdrB3KO MS) was used. Thus, a minimum sample of 5 mice per group (20 mice in total) was necessary to detect differences in means at a significance level of 5% with a 95% power in 1-way ANOVA, regarding the differences in the percentage of time spent on each object (known and new). For this, the values of 153.13 and 127.20 were considered, respectively, for the mean square between groups and intragroups.

The experimental data were analyzed using PRISM software (GraphPad Software, San Diego, California USA). The Shapiro-Wilk test was adopted to verify normality among data. For all analysis of the statistical significance of the differences among the mean values for the groups 2-way ANOVA was used, followed by Bonferroni's multiple comparisons test, and a p value <0.05 was considered statistically significant.

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