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Orexin/hypocretin treatment restores hippocampal-dependent memory in orexin-deficient mice

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Abstract

Orexin A is produced in neurons of the lateral, perifornical and dorsomedial regions of the lateral hypothalamic area, which then project widely throughout the central nervous system to regulate arousal state, sleep-wake architecture, energy homeostasis and cognitive processes. Disruption of orexin signaling leads to sleep disturbances and increased body mass index, but recent studies also indicate that orexin neuron activation improves learning and memory. We hypothesized that hippocampal orexin receptor activation improves memory. To test this idea, we obtained orexin/ataxin-3 (O/A3) mice, which become deficient in orexin neurons by about 12 weeks of age. We first measured hippocampal orexin receptor 1 (OX1R) gene expression and protein levels, then tested acquisition and consolidation of two-way active avoidance (TWAA) memory, a hippocampal-dependent learning and memory task. Finally, we determined if exogenous intra-hippocampal OXA treatment could reverse cognitive impairment (as determined by TWAA) in O/A3 mice. We showed that OX1R mRNA expression and protein levels were significantly elevated in O/A3 mice, indicating the potential for preserved orexin responsiveness. The O/A3 mice were significantly impaired in TWAA memory vs. control mice, but OXA treatment (both acute and chronic) reversed these memory deficits. These results demonstrate that orexin plays an important role in hippocampal-dependent consolidation of two-way active avoidance memory, and orexin replacement can rescue the cognitive impairment.

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Keywords

Hypocretin; Orexin ataxin-3; Orexin 1 receptor

Introduction

The peptide orexin A (OXA), produced in lateral hypothalamic neurons, is known to facilitate neuronal modulation of autonomic and behavioral functions such as feeding, physical activity drive, and sleep/wake states [1,2]. The total number of orexin neurons is small, but projections extend throughout the brain and spinal cord [3], including hippocampus [4,5], and distribution of neurons and projections is similar across species [6]. Orexin A has also been found to modulate neural plasticity, cognition, learning, and memory [7,8,9,10,11,12]. Orexinergic stimulation can increase neurogenesis in the hippocampal dentate gyrus, suggesting a prominent mechanism for cognition promoting effects of orexin [13]. In hippocampus, OXA can activate CA1 neurons, cells critical in long term memory, via orexin 1 receptor (OX1R) [12]. Separate reports have demonstrated that OXA increases long-term potentiation (LTP) in the CA1 area of the hippocampus, while orexin receptor antagonism decreases performance in progressive and variable ratio operant testing, as well as spatial memory acquisition and consolidation [10,14,15,16,17]. In a rodent model of AD that overproduces amyloid beta (SAMP8 mice), orexin improves active avoidance memory [18]. Deficiencies in OXA signaling due to orexin cell loss are linked to sleep disturbances, neurodegeneration, and cognitive impairments in humans and animals [7,19,20,21,22,23,24]. Clinically, altered OXA level in cerebrospinal fluid (CSF) is associated with learning and memory impairments [25], and reduced CSF OXA is considered a definitive diagnostic test for narcolepsy [26]. Paradoxically, Alzheimer's disease (AD) patients diagnosed with nocturnal sleep disruption present with increased CSF levels of OXA, potentially due to a compensatory mechanism of orexin neuron degeneration [27]. These data indicate that in addition to known effects on sleep/wake, altered orexinergic signaling can directly impair memory and cognitive function.

The orexin/ataxin-3 (O/A3) mouse is a genetic model of selective neurodegeneration in which the toxic protein ataxin-3 is expressed only in orexin cells, leading to progressive loss of these neurons [28]. Initial characterizations showed that O/A3 mice have normal levels of orexin neurons at birth, but lose over 50% of these neurons by 2 weeks of age, and 99% by 12 weeks, with no loss of other hypothalamic neurons, indicating a specific and gradual loss of these neurons during development. [28]. Because O/A3 mice express normal levels of orexin and orexin-responsive neurons during development, they are more likely to exhibit normal response to exogenous orexin than would a global knockout animal. In addition, the loss of orexin neurons themselves (rather than knock-down of the orexin peptide) mimics the loss of orexinergic neurons observed in neurodegenerative diseases [7,14,19,20,21,29,30].

In the current study, we hypothesized that activation of dormant hippocampal orexin circuits could counter orexin loss effects on cognition in O/A3 mice. To test our hypothesis, we measured cognition using a two-way active avoidance test in O/A3 mice. We further tested whether exogenous orexin rescues these behavioral deficits in O/A3 mice.

Methods

Animals

Six-month old female O/A3 mice were obtained from our breeding colony at the Minneapolis VA Health Care System. The colony originated from founder animals obtained from Dr. Masashi Yanagisawa at the University of Texas-Southwestern, bred to wild-type C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME USA). Genotype was confirmed using PCR as described in [28]. Mice were maintained in group housing (or single housing, during behavioral testing) under a 12:12 hour light/dark cycle (lights on at 0600 h) in a temperature controlled room at 21–22°C. Teklad 8604 Lab Chow (Harlan, Indianapolis, IN USA) and water were provided *ad libitum*. Body weights were monitored during all tests, and body composition was recorded using an EchoMRI 700 (Echo Medical Systems, Houston, TX USA) as previously described [31]. The experimental protocol was approved by the local Institutional Animal Care and Use Committee.

Reverse transcription polymerase chain reaction (qRT-PCR)

Hippocampal tissue was micro-dissected as described in [32,33] and RNA was isolated with the aid of Trizol reagent. Real-time thermal cycling were carried out in a Roche LightCycler (Roche Diagnostics Corporation, Indianapolis, IN) by one-step RT-PCR using the general method as previously described [34]. The following genes were analyzed: OX1R (NM_198959) and GAPDH (NM_017008). Relative mRNA levels were normalized to GAPDH using the 2^{-CT} method [35], and analyzed using an unpaired t-test (GraphPad Software, La Jolla, CA USA).

Western Blots

Hippocampal tissue was micro-dissected and protein extraction was performed using a RIPA commercial protein extraction kit (Thermo Fisher Scientific, Rockford, IL) [33]. Protein quantification was determined using a Bradford assay (BioRad, Hercules, CA) and samples (10 µg per well) were separated on 10% Criterion TGX gel (BioRad) and then transferred to PVDF membrane, and blocked (Super Blocker; Thermo Fisher Scientific) using Snap ID system (Millipore, Billerica, MA). Antibodies: OX1R (ab68718, Abcam, Cambridge, MA) and GAPDH (NB100-56875; Novus) primary; HRP conjugated anti-rabbit IgG (NB710H, Novus, Littleton, CO) secondary. All primary antibodies were used at a 1:1000 dilution, while secondary antibodies were used at a final dilution of 1:60,000. Protein samples were visualized and band density was determined (LICOR Image Studio Lite 3.1, Lincoln, NE). Data were normalized to GAPDH [36,37], and analyzed using unpaired t-test using GraphPad Prism.

Two-Way Active Avoidance (TWAA) Task

As shown in Fig. 1A, mice were placed in the automated two-way shuttle box (size, 45.7 × 20.3 × 30.5 cm; model 75-FSFX-Fusion; AccuScan Instruments, Columbus, OH USA) and allowed to acclimate for 5 min. Mice could move freely throughout the shuttle box during acclimation and training trials. Mice were trained in a 20-trial shuttle box two-way active avoidance task. The procedures for the conditioned stimulus and unconditioned stimulus

were as follows. A tone (5000 Hz; 45 dB) and a pulsatile light (2.5 Hz) were presented as a conditioned stimulus in the compartment with the animal, paired 5 sec later with a 0.3 mA scrambled foot shock (unconditioned stimulus) delivered through the floor grid (steel rods 0.5 cm in diameter, spaced 1.5 cm between centers). The “scrambled” nature of the shock prevents the animals from finding a “no-shock” position on the floor. To avoid receiving a foot shock the mouse had 5 sec to move to the opposite compartment. If the animal did not move to the other compartment, the unconditioned stimulus was delivered for a maximum of 7 sec., and the conditioned stimulus ended with the unconditioned stimulus. While receiving the unconditioned stimulus, if the animal moved to the other compartment, both conditioned and unconditioned stimuli ended immediately. The inter-trial interval was variable with a mean of 40 sec. A computer using remote monitoring system software (Fusion software) was used to control experimental protocols and data collection [38,39,40]. The testing and treatment paradigm is shown in Fig. 1B. For baseline characterization, animals underwent an acquisition session and then testing sessions 24 h and 1 week post-training without receiving any treatment. The 24h and 1 week testing were performed to determine the group differences in short-term and long-term memory respectively in subsequent comparisons with the OXA-treated condition [41]. For studies of OXA treatment, immediately following TWAA baseline training (as described above), mice were injected one time with either aCSF or OXA, then tested for TWAA response (20 trials) 24 h later without additional OXA treatment. Next, mice were injected with either aCSF or OXA once daily for 6 days, and TWAA response (20 trials) was assessed again 24 h after final injection (without additional OXA treatment).

In an additional study, to address the acute effect of orexin on memory consolidation, mice were treated with OXA or aCSF immediately following TWAA baseline training (as described above), and tested for TWAA response (20 trials) 24 h later without additional OXA treatment. Next, mice were returned to their home cages and TWAA response (20 trials) was assessed again after 6 days (without additional OXA treatment).

An earlier study using O/A3 mice showed altered LTP, paired pulse facilitation, reduced pCREB levels in the CA1 area of the hippocampus, and impaired social recognition memory [14]. In addition, several reports indicate that orexin antagonism in the CA1 area impairs hippocampus dependent memory consolidation [17], whereas intra CA1 orexin enhances LTP in this region [14]. Accordingly, in the current study, we injected orexin into the CA1 area of the dorsal hippocampus, to test whether intra CA1 orexin restores TWAA task performance in O/A3 mice.

TWAA Data Analysis

To measure performance on the TWAA task, each trial was analyzed for successful avoidances, escapes and escape latency. Experimental data were analyzed using repeated measures (genotype x time) or two-factor (phenotype x treatment) ANOVA, followed by the Sidak or Bonferroni post-hoc analysis for multiple comparisons. In addition, a linear regression analysis was performed, after adjusting for the baseline cognitive performance. Significance was considered to be achieved where $p < 0.05$. Analyses were performed to determine, a) the differences in acquisition between phenotypes, and b) differences in

retention between treatment groups. The improvement of performance between training trials (first session) and test trials (all sessions thereafter) were analyzed for avoidances (crossing after conditioned stimulus, but before shock delivery) escapes (crossing to opposite side during shock delivery), total responses (avoidances + escapes) and for escape latency (interval between conditioned stimulus and a response). These parameters were also compared between groups during test sessions to see if there was any specific effect of treatment (aCSF vs. OXA) on memory retention. Statistics were performed using GraphPad Prism [39,40].

Spontaneous physical activity (SPA)

SPA, defined as total physical activity in x, y, and z axes, was measured using clear acrylic chambers (43.2 cm × 43.2 cm × 30.5 cm) equipped with infrared beam arrays to capture movement in x, y, and z axes (Med Associates, St. Albans, VT USA). Mice remained in the chambers for a total of 48 h (24 h to acclimate, followed by 24 h of activity monitoring). Animals had *ad libitum* access to food and water while in the chambers. Chambers were cleaned and fresh food was added at the end of the acclimation period. Time spent moving in horizontal and vertical axes were analyzed using two factor ANOVA (genotype x time) using GraphPad Prism.

Surgery

Mice were anesthetized with isoflurane mixture (3% for induction, and 1.5% for maintenance), and implanted with bilateral 26-gauge stainless steel cannula (Plastics One, Roanoke, VA USA) directed towards the cornu ammonis area-1 (CA1) of dorsal hippocampus. Stereotaxic coordinates for the CA1 area (lateral ± 1.3 mm; anterior/posterior -1.3 mm; dorsal/ventral -1.7 mm; all measurements relative to bregma) were determined using a mouse brain atlas [42]. The injector extended 0.2 mm beyond the tip of the cannula. Animals recovered for 10 days, and were individually housed during recovery and testing.

Orexin A Injections

Prior to OXA injections, animals were acclimated to the restraint and injection procedure with three days of artificial cerebrospinal fluid (Harvard Apparatus) infusion. The infusions of either artificial cerebral spinal fluid (aCSF, Harvard Apparatus, Holliston, MA USA) or OXA (American Peptide Co., 250 pmol/0.2 μ l per site) were made daily using a KD Scientific automatic syringe pump (KD Scientific Inc., Holliston, MA USA) coupled to a Hamilton syringe (Series 7000, Sigma-Aldrich, St. Louis, MO USA). The animals were gently scruffed to place the bilateral injector into the guide cannula and injectate was delivered into both hemispheres simultaneously over 1 minute. All injections occurred between 1000–1130 h [2].

Results

Characterization of the O/A3 phenotype

We characterized baseline differences between orexin-deficient and wild type mice, using animals produced in our breeding colony. Consistent with prior published data, O/A3 mice

in our colony weigh more and are less physically active than age matched wild type animals (data not shown). The physical activity difference is limited to the dark (active) phase, as in an open field activity test. Post hoc analysis showed that while 6-month-old O/A3 males were less active than wild type males during the dark phase ($p = 0.0026$), they were not significantly less active than wild type males during the light cycle phase (2 way repeated measures ANOVA (genotype x time) with Sidak's multiple comparison test; genotype $F_{(1,14)} = 6.515$, $p = 0.0230$; time $F_{(1, 14)} = 6.249$, $p = 0.0255$; interaction $F_{(1, 14)} = 42.34$, $p < 0.0001$; Fig. 2). As shown in Fig. 3A and B, OX1R RNA expression and protein is increased in O/A3 mice relative to wild type littermates OX1R ($p < 0.01$).

Orexin loss causes cognitive impairment

We tested performance of O/A3 ($n = 9$) and control mice ($n = 9$) in a hippocampal-dependent two-way active avoidance (TWAA) task. This test is thought to measure both long- and short-term hippocampal-dependent memory [38]. Animals are exposed to a conditioned stimulus (light and sound) followed by a foot shock (unconditioned stimulus) in one compartment of a two-compartment shuttle box. Avoidance or escape from foot shock, or reduced latency to do either, is considered a measure of learning. Twenty-four hours after initial training (20 trials of TWAA), the animals underwent testing (20 additional TWAA trials) to assess learning. While initial training performance did not significantly differ between the groups, repeated measures ANOVA showed a significant effect of genotype and significant interaction between genotype and time of test for both escape latency (main effect $F_{(1,16)} = 4.78$; $p = 0.0439$; interaction $F_{(2, 32)} = 11.32$, $p = 0.0002$) and total response (main effect $F_{(1,16)} = 8.02$; $p = 0.0120$; interaction $F_{(2, 32)} = 17.76$, $p < 0.0001$) (Figs. 4 and 5). Specifically, Sidak post-hoc tests showed that the O/A3 mice exhibited significant impairments in TWAA memory vs. control mice at 24 hours post-training (higher response latency, $p < 0.01$; reduced total response (avoidance + escapes), $p < 0.01$) and one week after initial training (higher response latency, $p < 0.01$; reduced total response, $p < 0.001$) vs. control mice. Importantly, O/A3 mice continued moving within the same compartment as the shock, in response to the stimulus, rather than crossing to the opposite compartment, indicating absence of TWAA memory. Together these results indicate that orexin loss impairs hippocampal-dependent memory retention.

Intra-hippocampal OXA treatment rescues memory impairment in orexin/ataxin-3 mice

Based on the finding that O/A3 mice have significant impairments in the TWAA memory, we sought to determine if dorsal hippocampus injection of OXA reverses observed deficits. O/A3 ($n = 13$) and control mice ($n = 10$) were trained with 20 trials of the TWAA task, followed immediately by bilateral injections of aCSF or OXA (250 pmol/side in 0.2 μ l aCSF) into CA1 region of dorsal hippocampus. Twenty-four hours after the injections (with no additional OXA), animals underwent testing (20 additional TWAA trials) to assess retention of learning. We found a significant effect of phenotype on latency ($F_{(4, 18)} = 19.41$; $p = 0.001$), avoidance ($F_{(4,18)} = 4.0$; $p = 0.01$) and total response ($F_{(4,18)} = 19.25$; $p = 0.001$) for aCSF-treated O/A3 animals. While initial training performance did not significantly differ between the groups (Fig. 6–A and B; Fig. 7), the O/A3 mice treated with vehicle (aCSF) showed significant impairments in TWAA learning when tested 24 h after the training (higher response latency, $p < 0.01$; lower avoidances, $p < 0.01$; reduced total

response (avoidance + escapes), $p < 0.05$) (Fig. 6C–D; Fig. 7). A significant phenotype $\times$ treatment interaction indicated an effect of OXA treatment in O/A3 mice on latency ($F_{(4,18)} = 19.41$; $p = 0.001$), avoidance ($F_{(4,18)} = 4.0$; $p = 0.01$) and total response ($F_{(4,18)} = 19.25$; $p = 0.001$). OXA treatment significantly improved TWAA task learning in the 24 h test among O/A3 mice, and post-hoc analysis showed that OXA-treated O/A3 mice exhibited lower response latency ($p < 0.001$), higher escapes ($p < 0.01$), higher avoidances ($p < 0.05$), and increased total response ($p < 0.001$) compared to aCSF-treated O/A3 mice.

Next, we tested whether long-term treatment with OXA normalizes learning impairments observed in the O/A3 mice. To do this, after the 24 h testing session, the O/A3 mice were treated with once daily injections of orexin or aCSF for one week, which was followed by a TWAA test 24 h after the last injection (without additional OXA). We demonstrate a significant effect of phenotype on latency ($F_{(4,18)} = 22.08.41$; $p = 0.001$), escapes ($F_{(4,18)} = 3.23$; $p = 0.01$) and total response ($F_{(4,18)} = 38.66$; $p = 0.001$) for aCSF treated O/A3 animals. There was significant interaction between phenotype and treatment, indicating an effect of OXA treatment in O/A3 mice on latency ($F_{(4,18)} = 22.08$; $p = 0.01$), escapes ($F_{(4,18)} = 3.23$; $p = 0.05$) and total response ($F_{(4,18)} = 38.66$; $p = 0.001$). Similar to 24 h testing, learning impairment in aCSF treated O/A3 mice was evident one week after initial training (higher response latency, $p < 0.001$; fewer escapes, $p < 0.05$; reduced total response, $p < 0.001$) vs. aCSF treated control mice (Fig. 6E–F; Fig. 7). The O/A3 mice treated with OXA showed significant improvements in TWAA task learning vs. vehicle treated O/A3 mice (lower response latency, $p < 0.001$; higher escapes, $p < 0.05$; higher avoidances, $p < 0.05$; increased total response, $p < 0.001$). Additionally, OXA increased the number of avoidances in both WT and O/A3 mice, the effect being statistically significant in O/A3 mice (Fig. 7).

Finally, we tested whether a single intra-hippocampal OXA injection acutely enhances memory consolidation. To do this, immediately after the baseline training session, animals were treated with either aCSF or OXA. Twenty four hours after the injections, mice were tested for TWAA task performance. After this, animals were returned to their home cages and allowed to rest for 6 days. On the seventh day, mice were again tested for TWAA performance (without additional OXA). While initial training performance did not significantly differ between the groups (Fig. 8-A and B), the O/A3 mice treated with vehicle (aCSF) showed significant impairments in TWAA learning when tested 24 h after the training (lower escapes, $p < 0.05$; reduced total response (avoidance + escapes), $p < 0.05$) (Fig. 8C–D). A significant phenotype $\times$ treatment interaction indicated an effect of OXA treatment in O/A3 mice on number of escapes and total response. OXA treatment significantly improved TWAA task learning in the 24 h test among O/A3 mice, and post-hoc analysis showed that OXA-treated O/A3 mice exhibited higher escapes ($p < 0.01$), and increased total response ($p < 0.001$) compared to aCSF-treated O/A3 mice.

Similar to 24 h testing, learning impairment in aCSF treated O/A3 mice was evident one week after initial training (fewer escapes, $p < 0.05$; reduced total response, $p < 0.05$) vs. aCSF treated control mice (Fig. 8E–F). The O/A3 mice treated with OXA showed significant improvements in TWAA task learning vs. vehicle treated O/A3 mice (higher escapes, $p < 0.05$; increased total response, $p < 0.01$).

Together these results indicate that loss of orexin signaling impairs hippocampal-dependent learning, and that OXA rescue treatment (acute or long-term) reverses learning impairments in the O/A3 mice. The results indicate that OXA treatment plays a key role in consolidation and maintenance of hippocampal based memory.

Discussion

In our present study, we tested OXA in hippocampal-dependent learning and memory by evaluating TWAA performance impairment in OA3 mice. Earlier studies showed that the TWAA task is dependent on integrity of the dorsal hippocampus [39,40]. We now demonstrate that OXA treatment in the hippocampus rescues cognitive deficits in orexin deficient O/A3 mice as demonstrated by performance in the TWAA task. In addition, our acute OXA injection study indicates that OXA enhances TWAA performance by modulating memory consolidation (Fig. 8). This finding accords with earlier gain of function studies, which have shown improved task performance following OXA administration in animals [8,9,14]. Orexin neurons project to many brain sites important in cognition, executive function, learning, and memory, and several studies indicate that orexin release in these areas contribute to modulation of learning and memory [6,14,17]. Orexin appears to exert a neuroprotective effect [37] and orexin neuron loss occurs during progression of neurodegenerative diseases including narcolepsy, Parkinson's, Alzheimer's and Huntington's disease [7,19,21,30]. Loss of orexin neurons in neurodegenerative diseases may predispose the brain to subsequent insult. We showed that mice with progressive orexin neuron loss are more prone to develop cognitive deficits than wild type animals (Figs. 4 and 5). While O/A3 mice lose orexin with age, we show for the first time that hippocampal orexin receptor expression is unchanged in these animals (Fig. 3), and they remain responsive to exogenous orexin as indicated by improved cognition (Figs. 6, 7 and 8) after repeated (Fig. 7) or just one injection of OXA during memory consolidation (Fig. 8). We are the first to demonstrate that orexin receptor levels do not decline in response to orexin loss, and are rather increased in O/A3 mice (Fig. 3). This may be a compensatory mechanism to ensure that any available orexin input activates responsive hippocampal neurons, but this prediction has not yet been tested.

Our current data and other published reports show that exogenous orexin can reverse cognitive deficits due to orexin loss in O/A3 mice [43,44]. Prior data indicate that orexin is involved in modulating long-term synaptic plasticity in hippocampus, a cellular mechanism underlying learning and memory [45]. In O/A3 mice, OXA injection targeting the CA1 and dentate gyrus improves long-term potentiation (LTP) [9,14,46]. At least one study has demonstrated that orexin is critical for the consolidation of hippocampal-dependent social memory, and that exogenous orexin normalizes this impairment [14]. In rodent and nonhuman primate studies, exogenous OXA improves cognitive processes, passive avoidance memory, spatial memory, contextual fear conditioning, and operant task performance [8,9,10,11,15,47,48]. Several studies have demonstrated that selective antagonism of OXA receptors in the CA1 region of hippocampus impairs memory in rodents [16,17]. Chronic orexin receptor blockade in rats reduces both the development and persistence of generalized avoidance [49]. In rats, OXA administration increases firing rate of CA1 neurons via activation of OX1R [12], and spatial and passive avoidance memory is

impaired following administration of the OX1R antagonist SB-334867-A into the CA1 and dentate gyrus [17]. In addition, blockade of CA1 OX1Rs in the expression phase disrupted morphine sensitization-induced memory restoration in rats [16], further supporting the role for OX1R in hippocampal-dependent memory consolidation. Therefore, it is likely that orexin plays an important role in learning and memory by acting on OX1R [17]. Experiments evaluating the role of orexin in hippocampal-dependent memory show that response is altered when orexin signaling is dampened [45]. Memory impairment in orexin deficiency is also supported by human studies. For example, narcoleptic patients often exhibit disease-related deficient memory functions [50,51] while other human data indicates dampened OXA levels associated with social interaction, a task dependent on hippocampus [52].

The performance of WT and O/A3 animals were identical during the acquisition phase in the current study. This suggests that orexins interact with the CA1 neurons of the dorsal hippocampus to process the storage of learned information, but may not be critical in the stages of encoding (e.g., acquiring the information) (Figs. 4 and 5). Reduced TWAA performance in O/A3 mice during testing is not due to a generalized decrease in motor behavior. As shown in Fig. 2, the total locomotor activity during the light period (during which the TWAA test was performed) did not differ between WT and O/A3 mice. In addition, while cataplexy was observed in some O/A3 mice during the dark phase in other experiments, we did not observe any cataplexy in the O/A3 mice during TWAA testing, perhaps due to the absence of total loss of orexin neurons in these mice at the time of testing. Increased TWAA performance after OXA treatment is not likely due to increased orexin-induced arousal, as all tests were performed at least 24 h after the last orexin injection, and the OXA waking effect lasts only 2 h post injection in rats [53,54].

One possible mechanism for the absence of TWAA memory consolidation in O/A3 mice is impaired synaptic plasticity due to deficient input from orexin neurons [14]. A recent study showed that, despite normal basal neurotransmission, the CA1 area of O/A3 mice had lower levels of phosphorylated CREB (pCREB), paired-pulse facilitation, and LTP [14], indicating reduced short- and long-term synaptic plasticity. OXA stimulation in hippocampal slices from O/A3 mice restored pCREB and LTP. Restoration of pCREB activity by OXA was necessary to retain consolidation of social recognition memory [14]. Formation of long-term memory in hippocampal-dependent cognitive tasks depend on CREB phosphorylation, which is critical for memory storage and sustained hippocampal synaptic plasticity [55]. These data suggest that exogenous orexin reverses memory impairments in O/A3 mice in part by enhancing LTP, CREB phosphorylation, and synaptic plasticity in the hippocampus [14]. In addition, activation of OXA receptors also leads to stimulation of protein kinase A, C and calcium calmodulin pathways which are involved in the modulation of synaptic plasticity [45,56]. Thus, orexin enhancement of CREB phosphorylation would enhance TWAA memory retention in O/A3 mice. Finally, it is possible that exogenous OXA might improve memory in O/A3 mice through induction of dentate gyrus neurogenesis, as several studies have demonstrated that orexin A induces neurogenesis in the dentate gyrus of rats and mice [13,46,57].

It is important to acknowledge that in addition to producing orexin, OXA neurons co-express neurotransmitters such as dynorphin, pentaxin, and glutamate, which may also influence memory [58,59,60]. Loss of orexin neurons thus also results in decreased signaling of these co-expressed neurotransmitters. We have not yet tested the extent to which exogenous administration of these transmitters affect TWAA performance. However, unlike orexin, these transmitters are produced in more than one cell type within the brain. Complete reversal of TWAA impairments by OXA alone in the current study support that lack of orexin, rather than absence of co-expressed neurotransmitters, causes cognitive impairments in the O/A3 mice.

In conclusion, the present study has demonstrated a role for orexin in processing of hippocampal-dependent TWAA memory. Orexin/ataxin-3 animals show impaired performance in TWAA memory, and just one injection of intra-CA1 orexin immediately after the TWAA task (period of memory consolidation) rescued the memory deficits. These data indicate that OXA treatment could be a potential therapeutic avenue for reversal of cognitive deficits associated with neurodegenerative diseases involving orexin neuron loss.

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Highlights

- Orexin 1 receptor expression is elevated in mice lacking orexin (O/A3 mice)
- O/A3 mice showed significant impairments in a two-way active avoidance memory task
- Memory deficits in O/A3 mice were reversed by hippocampal orexin A treatment

Figure 1.

A. Representation of the TWAA apparatus. Performance was analyzed for avoidances (crossing after conditioned stimulus, but before shock delivery), escapes (crossing to opposite side during shock delivery), total responses (avoidances + escapes), and escape latency (interval between conditioned stimulus and a response). **B. Experimental design for cognitive testing and OXA treatment.** For studies involving OXA treatment, immediately following completion of training, mice were given a single dose of OXA or aCSF. Mice were retested for TWAA response 24 h later without additional OXA. Next, mice were given OXA or aCSF once daily for 6 days prior to the final TWAA test, with 24 h between the final dose and time of test.

Figure 2. Open field activity in O/A3 and wild type mice

Orexin/ataxin-3 (O/A3) mice show significantly less dark phase physical activity (ambulatory + vertical time) than wild type (WT) animals. However, light phase physical activity is identical between groups. 2-way ANOVA; significant effect of phenotype ($p = 0.0352$); Significant interaction (time $\times$ phenotype; $p < 0.0001$). * $p < 0.05$. $N = 8/\text{group}$.

Figure 3. Orexin receptor-1 in hippocampal tissue is upregulated in O/A3 mice

(A) Gene expression for OX1R is upregulated in hippocampal tissue in O/A3 vs. WT mice.

(B) Orexin receptor-1 protein levels are increased in O/A3 vs. WT mice. Data are represented as mean $\pm$ SEM. (N = 5 per group, ** p < 0.01 O/A3 vs. WT).

Figure 4. Orexin loss in O/A3 mice increases latency and decreases total response

Performance on TWAA task is reduced in O/A3 female mice at 24 h and 1 week post-training. Latency and the total response (avoidance + escape) did not differ in control and O/A3 mice during task training. Tests performed 24 h and 1 week post-training both revealed impaired memory in the O/A3 mice, as indicated by higher latency, and decreased total responses. $N = 9$ for WT, and $N = 9$ for O/A3 mice. Data are represented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ for between group comparisons.

Figure 5. Orexin loss in O/A3 mice reduces escapes and avoidance

Performance on TWAA task is reduced in O/A3 mice at 24 h and 1 week post-training.

Number of escapes and avoidance did not differ in control and O/A3 mice during task training. Tests performed 24 h and 1 week post-training both revealed impaired learning in the O/A3 mice, as indicated by reduced escapes (1 week; A), and decreased avoidances (24 h; B). $N = 9$ for WT, and $N = 9$ for O/A3 mice. Data are represented as mean $\pm$ SEM. ** $p < 0.01$ for between group comparisons.

Figure 6. Orexin treatment in O/A3 mice normalizes escape latency and total response
 Performance on TWAA task is reduced in O/A3 mice at 24 h and 1 week post-training. Orexin treatment reversed these impairments in TWAA memory. Latency and total responses did not differ in control and O/A3 mice during task training. Tests performed 24 h and 1 week post-training revealed impaired learning in the O/A3 mice, as indicated by higher latency, and decreased total responses. Orexin treatment reversed the learning impairments in O/A3 mice after single injection (24 h post-test) and after daily injections for a week (1 week), as indicated by lower latency, and increased total responses. N = 10/group for WT

and N = 13 for O/A3 mice). Data are represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 for O/A3 + aCSF vs. O/A3 + OXA comparisons. #P < 0.05 for WT vs. aCSF comparisons.

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Figure 7. Orexin treatment in O/A3 mice normalizes escape and avoidance behavior

Number of escapes did not differ between groups during task training (A). Tests performed 1 week post-training revealed impaired learning in the O/A3 mice (O/A3 + aCSF), as indicated by decreased number of escapes compared to that in wild type animals (WT + aCSF; E). Long-term treatment of OXA (O/A3 + OXA; 1 time per day for 7 days; 1 week post training) reversed learning impairments in O/A3 mice, as indicated by increased escapes (E). Tests performed 24 h post-training revealed impaired learning in the O/A3 mice (O/A3 + aCSF), as indicated by decreased number of avoidances compared to wild type

animals (WT + aCSF; D). Single OXA treatment reversed the learning impairments in O/A3 mice (O/A3 +OXA; 24 h post training), as indicated by increased avoidances (D). N = 10/group for WT and N = 13 for O/A3 mice). Data are represented as mean $\pm$ SEM. * $p < 0.05$ vs. WT + aCSF comparisons. # $p < 0.05$ vs. O/A3 + aCSF comparisons.

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Figure 8. Acute orexin treatment in O/A3 mice normalizes number of escapes and total response Performance on TWAA task is reduced in O/A3 mice at 24 h and 1 week post-training. Orexin treatment reversed these impairments in TWAA memory. Number of escapes (A) and total responses (B) did not differ in control and O/A3 mice during task training. Tests performed 24 h and 1 week post-training revealed impaired learning in the O/A3 mice, as indicated by lower escapes (C, E), and decreased total responses (D, F). Single orexin treatment immediately after baseline training reversed the learning impairments in O/A3 mice observed during 24 h post-test and 1 week post-test, as indicated by higher escapes,

and increased total responses. $N = 6/\text{group}$ for WT and $N = 6$ for O/A3 mice). Data are represented as mean $\pm$ SEM. $*p < 0.05$ for O/A3 + aCSF vs. WT + aCSF comparisons.

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