The therapeutic effects of oxytocin on the autistic behavior induced by Valproic acid injection of pregnant BALBc mice mothers

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental syndrome that is marked by significant deficits in verbal and non-verbal social interactions, and by a peculiar pattern of restricted repetitive behaviors. Oxytocin is known for its involvement in mammalian social behavior and probably it might improve the symptoms of ASD. This research investigated the effects of intraperitoneal oxytocin on offspring of valproate-injected mothers during pregnancy. Animals were divided into five groups: control group (Con), control mice treated acutely with oxytocin (Con Oxy), autistic mice received normal saline (Au NS), autistic mice treated acutely with oxytocin (Oxy Ac), and autistic mice treated with oxytocin for two weeks (Oxy 2wk). In an elevated plus maze test, Con animals showed no anxiety-like behavior while Au NS mice showed anxiety-like behaviors. However, there was a positive improvement in anxiety-like behavior in Oxy 2wk group. Animals that were treated with oxytocin showed a clear tendency for sociability and social novelty in the three-chamber test used to test social behavior. There was an increase in the parameters measured by the behavioral spectrometry, the average velocity, activity and ambulation in Au NS group. On the other hand, all parameters were decreased in both Au Oxy groups with significant changes in Oxy 2wk group. Our data conclude that oxytocin causes significant reduction in anxiety and improvement of social interaction and autistic behavior when it was administered for two consecutive weeks. In the contrast, short term exposure to Oxytocin probably showed no significance difference in the results, which is suggested to be attributed to the stress effect of the intraperitoneal injection as well as the possibility for needing more time for oxytocin effect to be more prominent before testing.

Keywords: Autistic behavior, Mice, Oxytocin, Social behavior, Anxiety

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental syndrome that is marked by significant deficits in verbal and non-verbal social interactions, and by a peculiar pattern of restricted repetitive behaviors [1]. Over 70% of individuals who suffer from ASD present with comorbidity [2]; the concurrent conditions that are commonly associated with ASD include clinical depression, attention-deficit hyperactivity disorder (ADHD), epilepsy and gastrointestinal (GI) problems [3]. Although ASD can occur in any individual, irrespective of race, ethnicity or socioeconomic status, statistics have shown that ASD is around 4.5 times more common among male than female individuals [4]. In addition, various research studies have demonstrated a significantly progressive rise in the incidence of ASD over the last few decades [5]. This was relatively attributed to a “Western Style” diet [5]. In spite of this positive correlation, the etiology of ASD remains multifactorial, encompassing environmental factors such as prenatal exposure to valproic acid [6], genetic factors such as fragile-x-syndrome and biological factors such as low plasma oxytocin levels [7,8].

Evidence concerning the therapeutic approach of oxytocin in ASD individuals is limited; however, researchers continue to investigate the relative uses of oxytocin in improving the signs and symptoms of ASD. Oxytocin (OT) is a peptide hormone consisting of 9 amino acids. It is released into the bloodstream through axon terminals of the posterior pituitary; however, its site of synthesis is within the paraventricular and supraoptic nuclei of the hypothalamus [9].

OT is crucial in facilitating uterine contractions during childbirth and is also essential in the mechanism of milk let-down [9]. In addition, OT is known for its involvement in mammalian social behavior as it plays a relatively important role in regulating certain repetitive behaviors, such as those associated with social bonding, memory and recognition and is often referred to as the ‘love hormone’ [10]. Due to its nature
and involvement in controlling repetitive behaviors and social communication, researchers have been studying the suggested therapeutic aspect of oxytocin in decreasing the principal symptoms of ASD. The basis of this approach was on the relative association between variants in the oxytocin receptor gene and ASD, revealed by a significant number of studies. One of which illustrated the implication of the rs53576 variant in social-behavioral phenotypes and social impairment [11]. As a result, other research studies have concluded that emotional recognition in autistic individuals can be improved by the use of oxytocin nasal spray [12,13]. Similarly, another research suggested that oxytocin may increase social cognition [14]. Although there is minimal evidence to support the safety and efficacy of oxytocin use to treat ASD, additional animal studies investigating the role of oxytocin on autistic models of mice have shown a slightly yet unclear positive correlation, suggesting the importance of conducting further investigations regarding this phenomenon [15].

This research investigates the effects of intraperitoneal oxytocin on the male mice with autistic-induced behavior.

Materials and Methods

Animals

BALB/c male mice, bred in the animal department in Arabian Gulf University were used. They were housed in cages on sawdust and had access to food and water ad libitum. Animal care and experimental ethics were applied according the Arabian Gulf University guidelines for the use of animals in experiments. The research was conducted in five groups; each containing 8 male mice as follows;

1. Control group A (Cont) contained normal mice offspring that were given normal saline only.
2. Experimental group A (Cont Oxy) included normal mice offspring were exposed to oxytocin.
3. Control group B (Aut NS) contained VPA exposed mice offspring that were given normal saline only.
4. Experimental Group B1 (Oxy 2wk) included VPA exposed offspring that were treated with oxytocin for 14 days before testing.
5. Experimental Group B2 (Oxy Ac) included VPA exposed offspring that were treated with oxytocin 30 minutes before testing.

Virgin female mice at the age of 10-12 weeks were mated with male mice overnight (1-2 female: male) to establish a pregnancy; that was confirmed by the presence of a vaginal plug in the following morning which was designated as the 1st embryonic day E1. The animals were tested on postnatal days 44-49.

Drugs

1. Sodium salt of valproic acid (NaVPA, Sigma-Aldrich) was prepared in 0.9% saline (100 mg/ml, pH 7.3), and a single intra-peritoneal (IP) injection of NaAVP (600 mg/Kg) was administered on embryonic day 12.5 E12.5. The control groups received a single IP injection of saline vehicle (3.3 ml/Kg) [17].
2. Synthetic oxytocin (Sigma-Aldrich) was dissolved in normal saline to give a concentration of 0.1 mg/ml and a dose of 1 mg/Kg of OT was given as an intraperitoneal injection over four testing days. The groups that was treated with OT received saline IP injection instead. The behavioral experiments were performed 30 minutes post-injection by which time the OT brain levels would have peaked.

Tests

Behavioral tests were conducted between 12:00 PM and 5:00 PM and were performed and scored blindly.

Three chambers social apparatus (Crowley’s sociability and preference for social novelty test): The three-chamber social apparatus was used to assess the sociability and preference for social novelty [16] [17]. The apparatus is comprised of a rectangular, three- chambered box. Each chamber is 20 cm × 40 cm × 22 cm. the walls are made of clear Plexiglas. The dividing walls made from same material, have small square openings (5 cm × 3 cm) allowing access into each chamber. Each chamber contains a circular wire cage that is 11 cm high, with a bottom diameter of 9 cm and bars spaced 0.5 cm apart. The subject mouse was first placed in the middle chamber to habituate for five minutes. The first session was started when an unfamiliar male mouse (stranger 1), that had no barrier contact with the subject mouse, was placed inside the wire cage in one of the side chambers. The subject mouse was allowed to explore the entire apparatus freely. The placement of stranger 1 in the left or right side chamber was systemically alternating between trials. The first session was continued for 10 minutes, and the time spent in each chamber and the number of chamber entries was recorded. Session 2 was started immediately after the end of session 1, with a second unfamiliar mouse being placed in the wire cage inside the chamber that had been empty during the first session. The test mouse had to choose between the chamber containing the already investigated mouse (stranger 1), and the one containing the novel unfamiliar mouse (stranger 2). The same parameters recorded for session 1 was recorded for session 2. The apparatus was cleaned with 70% ethanol and water between subjects. Three trails were done for each mouse.

Session 1 tests for sociability (or social motivation/ affiliation), which is spending significantly more time in the chamber containing a mouse than in the empty chamber. Session 2 tests for preference for social novelty, which is spending significantly more time in the chamber containing the novel mouse than the one containing the already investigated mouse [18].

Elevated Plus Maze Test

The elevated plus maze tests for anxiety-like behavior [16]; it consists of two open arms (25 cm × 5 cm) and two enclosed
arms of the same size with 15-cm-high walls. The arms are elevated 55 cm above the floor. To minimize the likelihood of animals falling from the apparatus, 3-mm-high walls surrounded the sides of the open arms. Arms of the same type are located opposite from each other. Each mouse was individually placed in the central square of the maze (5 cm × 5 cm), facing one of the closed arms and was allowed to freely explore the apparatus. Mouse behavior was recorded during a 10-min test period. The number of entries into an arm and the time spent in the open and enclosed arms were recorded. Percentage of entries into open arms, time spent in open arms (s), and total number of entries was analyzed. Entering the open arms less frequently and spending less time in them are indicative of anxiety-like behavior. The apparatus was cleaned with 70% ethanol between subjects. An open or closed arm entry is defined as all four paws in an arm. The numbers of open and closed arm entries were combined to yield a measure of total entries, which reflect general activity during the 10 min test.

Behavior Spectrometry

Different behaviors were assessed using behavioral spectrometer chamber (Behavioral Instrument, NJ, USA) (19x20 x 18 cm) equipped with floor, wall sensors and a single halogen light bulb that provide lighting of the field (~200-250 lux). Grooming and locomotor behaviors were determined by pattern recognition software that combines vibration, animal weight, and infrared beams.

Mice were placed in the center of arena and Data were collected over a 30-minute test session. Between sessions, the chamber was wiped with 70% ethanol.

Results

Elevated Plus Maze

The Elevated Plus Maze test aims to assess anxiety-like behaviors by recording the number of entries into the open arms, in addition to the cumulative time spent in each. A significant difference was recorded between the groups (ANOVA, P< 0.05, F 2.751, F critical 2.641, Figure 1).

Aut NS group showed an increased level of anxiety by means of spending significantly less time in the open arms, as opposed to the control group. (Control 104.1±22 VS Au NS 25±15.3, t-test P<0.005, t-critical=1.9). This, however, is almost remarkably reversed in the Oxy 2wk group, in which they distinguishably spent more time in the open arms (158.9±15.6, t-test P<0.001, t-critical 1.9). This is suggestive of a positive improvement in their anxiety-like behavior.

Three-Chamber Social Apparatus

The Three-Chamber Social Apparatus aims to assess sociability and preference for social novelty. This is done in two sessions, where the first session assesses sociability by recording the time spent in the chamber containing the mouse; and the second session assesses the preference for social novelty by recording the time spent in the chamber containing the new mouse, which was otherwise empty (in session one).

The control group evidently spent more time in the mouse-containing chamber than the empty chamber in session one (365.9 ± 45.8, t-test P<0.05, t-critical 1.9) hence, demonstrating normal sociability (Figure 2A). In session two, the control group also appeared to spend more time in the chamber containing the novel mouse, than that containing the former mouse (152.9 ± 22.5, t-test P<0.05, t-critical 1.9) hence illustrating a normal preference for social novelty.

Surprisingly, in session one, the Con Oxy group showed a considerable reduction in the tendency to socialize by spending more time in the empty chamber than that which contained the mouse (302.6 ± 92.4, 256.6 ± 86). In spite of these results, this group exhibited a normal preference for social novelty in session two by spending more time with the novel mouse than the former mouse (229.5 ± 79, 321.9 ± 77.9).

Aut NS group, showed a clear reduction in sociability in session one, as they had distinctly spent a less amount of time in the chamber containing the mouse (137.3±48.5), than that which was empty (355.7±125.7 , t-test P<0.05, t-critical 1.9). The sociability was significantly improved when mice were treated with oxytocin. Sociability was (289.3 ± 85.5) in Oxy Ac group; and (245.1 ± 42.4) in Oxy 2wk group.

When these groups were examined in session two, Aut NS group; and Oxy Ac group, illustrated little or no difference, in their preference for social novelty; hence, spending roughly the same amount of time in the chamber with the former mouse and the chamber with the new novel mouse. Respectively, the results were as follows: (233.2 ± 82.4) in the Aut NS group, and (203 ± 71.8) in the Oxy Ac group. Despite not being able to significantly revert the behavior of this latter group of mice to normal behavior, in terms of preference for social novelty, Oxy

Figure 1: Elevated plus maze test for the evaluation of anxiety in the different groups. Clear significant difference was measured between the Aut NS group and the other groups. Treating the animals either 30 min (Oxy Ac) or 2 weeks (Aut 2wk) before testing reversed the anxiety-like behavior into values not significantly different from the control.
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2 wk group showed a remarkable increase in the time spent with the new novel mouse (284.9 ± 41.7), as opposed to that spent with the former mouse (170.1 ± 39.1, Figure 2B).

There is a clear tendency for sociability and social novelty in the mice, which were injected with oxytocin.

Behavioral Spectrometry

The Behavioral Spectrometry allows one to study more than one aspect of behavior in animals by the simultaneous acquisition of more than one behavioral dimension; of which, we studied: the improvement of the Level of Activity and Velocity, Ambulation Behavior, Track Length and Zone Crossing (ZC).

Activity and Velocity

Levels of activity and velocity are fundamental factors in measuring the degree of autism-associated behaviors. In the context of this study, activity is measured as a velocity threshold, where the programmed velocity cut-off-point delineates active from inactive behavior.

The average velocity and percentage of activity were recorded; in the Con group were (3.8 ± 0.6, 9.4 ± 2.7) respectively; and (6.2 ± 0.7, 26.4 ± 6.6) in the Aut NS group. Evidently, a difference is noted; in which, a rise in values is significant to the latter group (Velocity: t-test P<0.05, t-critical 1.8), (activity: t-test P<0.05, t-critical 1.8, Figure 3A and B).

In addition, Oxy Ac group, demonstrated a slight decrease in the average velocity and percentage of activity (4.9 ± 0.9, 13.4 ±5) respectively.

However, Oxy 2 wk group demonstrated a remarkably significant decrease in the average velocity and percentage of activity (1.1 ± 0.4, 3.2 ± 1.3).

Track Length

This parameter measures the total length of the track traveled by the mouse in the whole arena. Significant difference was

Figure 2A: Three-chamber social test. The first session is testing the sociability of the animals. The animals from each group were given the choice to stay in a chamber with another animal, so called stranger 1 (so sociable) or in a chamber containing no animal (not sociable). The control animals showed the normal sociability while the Aut NS animals showed opposite behavior (preferred to stay more in the empty chamber). Treatment with Oxytocin reversed partially, although not completely this behavior.

Figure 2B: Social novelty was measured in session 2. The time spend by the animals in the chamber containing new animal (stranger 2) was compared with the time spent in the chamber containing the old animal from session 1 (stranger 1). Normal behavior, which is shown by the control group, was to stay more with the new animal (stranger 2). The Aut NS group showed no social novelty behavior, as they had no similar preference. Treatment with Oxytocin for two weeks could restore the normal behavior; but oxytocin treatment for 30 min before testing was not effective.

Figure 3A: The activity of the animals during 30 min of testing was recorded. It was the percentage of time the animals during which were active. The Aut NS group was significantly the most active. However, treatment with oxytocin for 30 min and 2 weeks were successful to decrease significantly this hyperactive states into comparable levels to the control group.

Figure 3B: The velocity of the animals during the 30 min of recording. The Aut NS group showed highest velocity compared to the others. Treatment with oxytocin for 2 weeks decreased significantly the velocity to lower levels, while treatment for 30 min was not effective.
recorded between the groups (ANOVA, P< 0.05, F 11, F critical 2.65). The average track length crossed by the Aut NS group, was higher (11191.2 ± 903.8) in comparison to the Con group (6725.1 ± 704.2, t-test P<0.05, t-critical = 1.8). Intra-peritoneal oxytocin injections revealed a significant reduction in the average track length. This was illustrated in the data acquired from the Oxy 2 wk group; in which their results showed much lower values (1927.4 ± 710, t-test P<0.05, t-critical 1.8) than the Aut NS group and less than half of the average length in the control group. Nonetheless however, Oxy Ac group, showed no significant difference in track length compared to the control group.

**Ambulation Behavior**

In behavioral spectrometry, ambulation behavior is defined as a spontaneous short-term acceleration. It is a function of an animal’s velocity. A significant difference was recorded between the groups (ANOVA, P< 0.05, F 5.1, F critical 2.65). As noted from the figure, Aut NS group showed a significant rise in ambulation when compared to the control group (Aut NS 5014.6 ± 1718.9 VS Con group 1204.4 ± 342.2, t-test P <0.05, t-critical= 1.8). Oxy 2 wk, had a significant reduction in ambulation (507.6 ± 214.7, t-test P <0.05, t-critical= 1.8) compared to Aut NS. Contrastingly, Oxy Ac group, had no significant differences when compared to the control group.

**Zone Crossing**

Zone crossing (ZC) is a parameter that reflects the effective number of line crossing events; it assesses open-field behavior in addition to the animal’s tendency to explore. A mouse with autistic-like behaviors such as anxiety-like behaviors would have a high ZC score.

Results shown by testing this parameter were significant (ANOVA, P< 0.05, F 3.3, F critical 2.65). Aut NS group, presented with the greatest ZC value (947 ± 180.3), compared to all other 4 groups; specifically greater than that of the Con group (672.6 ± 145.3) but the difference is not statistically significant.

Following the administration of oxytocin in the two remaining mice groups of valproate-injected mothers, the two groups illustrated a decline in Zone Crossing, where Oxy Ac group scored (818.4 ± 284.2), and Oxy 2 wk group scored (256.5 ± 94.4).

**Discussion and Conclusion**

In this study, we aimed at examining the therapeutic effects of Oxytocin on autistic model in mice. Our data clearly demonstrate that: (1) Valproic acid causes increased levels of anxiety as well as changes in behavioral aspects. It replicates the values of velocity, activity, ambulation and track length. (2) There is positive improvement in the anxiety like behavior with Oxytocin treatment. (3) There is a clear tendency for sociability and social novelty with Oxytocin injection. (4) Short term exposure to Oxytocin probably showed no significance difference in the results. The control mice group treated with oxytocin showed a reduced tendency for sociability. This could
be attributed to the stress effect of the intraperitoneal injection as well as the possibility for needing more time for oxytocin effect to be more prominent before testing. (5) There is a reduction in the parameters measured by the behavioral spectrometry, the average velocity, activity and ambulation influenced by Oxytocin.

One of the proposed hypotheses implicated in the development of autism was attributed to a reduction in the hypothalamic gray matter that is involved in the synthesis of oxytocin and vasopressin. This can be due to reduced number or size of neurons or neuropil compaction which are composed of glia, axons and dendrites, synapses, and blood vessel [19].

The role of brain synaptic pruning in the pathogenesis of autism spectrum disorder has been an area of great interest in recent studies. Post mortem examination of the brains of autistic children ranging between the ages of 13 to 20 years revealed universal mTOR over activity compared to normal children. This was associated with higher number of synapse density by late childhood in autistic brains. This reflects deficits in the synaptic pruning system which is responsible for eliminating inappropriate excess synapses as a child grows; a process referred to as “autophagy”. These excess synapses may be the cause of abnormalities seen in ASD [20,21].

In the three-chamber social apparatus test, the control mice exhibited normal pattern of sociability and preference for social novelty by spending more time in the chamber with the mouse than the empty chamber in the first session, and more time in the chamber with the novel mouse than the one with the familiar mouse in the second session, respectively. This behavior was impaired in Aut NS group by spending more time in the empty chamber in the first session and equal time investigating the novel and familiar mouse in the second session. The latter impaired preference for social novelty reflects abnormalities in social recognition and social memory. These results are in keeping with other studies which demonstrated impaired sociability and preference for social novelty in autism [22,23].

Mice of VPA mothers showed a tendency to restore sociability with acute and chronic OXT treatment; more evident in the former. This positive influence of OXT on social interaction in autism has been supported by several previous studies [24]. Of interest, two weeks IP OXT was able to restore the impaired social novelty in autism, while single OXT treatment had no impact. One hypothesis to explain this finding is that the influence on social novelty of the neuronal circuit may be cumulative and more clearly seen following long-term administration of OXT. Another explanation is the possibility of needing more time following injection for the maximum effect to be noted and for the stress of injection to wear off.

Unlike other study which confirmed the pro-social effect of peripheral OXT in the eusocial rats [25], the results in our study showed the opposite; the Con Oxy mice group spent more time in the empty chamber than the one containing the mouse suggesting lack of sociability. This unexpected finding can be attributed to the stress of the injection.

In attempt to assess anxiety, the elevated plus maze test was performed; measuring the level of anxiety in relation to the time spent in the open arm field. The Control group spent more time in the open arm indicating lack of anxiety. This was reversed in in Au NS group which exhibited anxiety like behavior by spending significantly less time in the open arm compared to the control. Interestingly, OXT significantly reduced the level of anxiety in mice of VPA mothers both acutely and chronically; the latter actually showing even better results than the Cont group. These findings are all in keeping with [24] study which demonstrated higher levels of anxiety in VPA rats which were reduced with early postnatal OXT treatment.

The prosocial effect of OXT is correlated to its anxiolytic action. The paraventricular nucleus PVN, one of the OXT production sites in the brain, is also responsible for the synthesis of the corticotrophin-releasing hormone CRH that stimulates the release of adrenocorticotropic hormone from the anterior pituitary. This activates the hypothalamic pituitary axis with subsequent release of cortisol from the adrenal gland [26]. It is noteworthy that a previous study examining the role of OXT on HPA axis showed transient rise of ACTH and corticosterone 30 minutes following injection with drastic drop 6 hours post injection [27]. Chronic OXT, on the other hand, caused a negative feedback on steroid release [28]. This in part may explain some of the inconsistencies found in the present study with the previous literature; the lack of sociability in Con Oxy and the failure to restore preference for social novelty acutely in Au Oxy.

The effect of single versus continuous OXT treatment in autism has been a debate of great controversy. Some studies surprisingly showed that chronic treatment of intranasal OXT was of less efficacy [29,30], our results on the contrary illustrated a more significant reduction in anxiety and improvement of social interaction and autistic behavior with two weeks IP OXT. This is in agreement with Dia et al. [24] which established long term behavioral effects on VPA rats with chronic early postnatal OXT administration, in correlation with restoration of OXT-ir cells in the PVN and SON.

Behavior is defined as “1. An organism’s activities in response to external or internal stimuli, including objectively observable activities, introspectively observable activities, and nonconscious processes; 2. More restrictively, any action or function that can be objectively observed or measured in response to controlled stimuli. Historically, behaviorists contrasted objective behavior with mental activities, which were considered subjective and thus unsuitable for scientific study.”

The control group in this research shows the normal behavior of the animals, to which the other groups can be compared. The offsprings from mothers injected with Valproic acid showed impaired behavior compared to the control group. These animals
exhibited a significant increase in all the behavioral spectrometry parameters that were tested; this, according to other studies, which had also studied animals exposed to VPA prenatally, is indicative of autistic behavior, owing to the increased levels of activity in their male [31] and female [32] animals.

Studies measuring the following parameters, which were tested in this study on prenatally VPA exposed animals (e.g. ambulation, track length, and zone crossing) may exist; however, no research was found to have used the behavioral spectrometry. This research may be the only research study that may have used the Behavioral Spectrometry to evaluate these parameters.

Although our study did not measure ambulation time per se, another study measured ambulation time of animals exposed to VPA prenatally, and no difference was seen [22]. This was also the case with our study when ambulation was assessed.

In addition, because zone crossing assesses exploratory behavior in mice. Therefore, it can be used as a measure of autistic-like behavior. On the other hand, researchers evaluated exploratory behavior in autistic-mouse models by using open-field testing as well. It was found that the trend for exploratory activity had lowered in the group of mice which were treated with oxytocin during the first 10 mins [12]. Similarly, in this study, zone crossing results in the mice of valproate-injected mothers, had evidently lowered after the injection of oxytocin 30 minutes prior to testing; and had substantially lowered after chronic injection of oxytocin for 2 consecutive weeks prior to testing. This therefore, essentially illustrates that oxytocin could be of great use in managing autistic-like behaviors.

On the other hand, there are two groups of VPA exposed animals prenatally. One of them was exposed to oxytocin 30 minutes prior to testing and exhibited a tendency for improvement in all the parameters. The other group was exposed to oxytocin for 2 weeks continuously, after which testing was conducted. The latter group showed much more significant results, proving that chronic exposure to oxytocin might be more effective in treating the monitored abnormal behavior in these animals. In addition, it was shown that nicotinic cholinergic system enhanced some of the parameters that were tested in this study. This effect was suggested to be due to upregulation of oxytocin receptors [33,34].

Overall, oxytocin showed a great tendency for improvement in autistic behavior in animals. The exact mechanism and through which this effect is achieved.

References
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