Postnatal Oxytocin Improves Survival and Long-term Neurodevelopmental Outcomes in an Animal Model of Neonatal Abstinence Syndrome

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ABSTRACT

Background: Neonatal abstinence syndrome (NAS) causes significant morbidity, is associated with costly and lengthy hospitalization and is increasing in prevalence. Evidence suggests neonates with NAS may have neurodevelopmental problems later in life. Current pharmacotherapy is suboptimal with no FDA approved treatments for NAS. We examined the effect of postnatal oxytocin (OT) treatment on survival and neurodevelopmental outcomes in rats prenatally exposed to opioids or benzodiazepines.

Methods: Sprague-Dawley rat dams were injected with escalating doses of morphine (10-50 mg/kg/day) or diazepam (2-15 mg/kg/day) throughout parturition. Rat pups thus exposed received subcutaneous injections of 2 mg/kg OT or saline for the first 10 postnatal days. Survival and bodyweight were measured. Another set of exposed rat pups received injections of saline or 0.3, 1, or 2 mg/kg OT or saline for the first 10 postnatal days and survival and body weight were measured for 30 days. Neurodevelopmental outcomes were assessed in animals surviving through adolescence.

Results: Postnatal OT treatment increased survival. OT improved long-term learning and memory processes while reducing behavioral signs of anxiety in animals prenatally exposed to morphine or diazepam.

Conclusions: These findings point to the potential of OT as a novel treatment for NAS. Clinical trials appear to be warranted.

INTRODUCTION

Background: Neonatal abstinence syndrome (NAS) is a constellation of signs of withdrawal caused by prenatal exposure psychoactive substances. Neonates with NAS experience central and autonomic nervous system dysfunction which if untreated can result in seizures and death (1-3). Emerging evidence also suggests that neonates with NAS have a higher incidence of neurodevelopmental problems later in life (4). The incidence of NAS has increased over fivefold in the US, affecting 8 neonates per 1000 births and 27 cases per 1000 NICU admissions (5). In 2012, the average length of hospital stay for neonates born with NAS was approximately 17 days resulting in an annual cost burden to hospitals of around $1.5 billion (7). No FDA approved treatments for NAS exist. Pharmacotherapy consists of opioid-based treatment (morphine, methadone, or buprenorphine). Due to the high rates of polysubstance use in women using opioids during pregnancy, adjunctive medications (e.g. clonidine, phenobarbital) are also used to manage NAS (8). While seizures and mortality rates have diminished substantially with pharmacological intervention, the current standard of care for NAS is suboptimal. In addition to the potential neurotoxic effects of opioid- and barbiturate-based treatments (9, 10), transitioning to homecare while neonates still require medications is problematic causing lengthy hospital stays and associated healthcare costs (11).

The neuropeptide oxytocin (OT) reduces naloxon e-induced morphine withdrawal in adult rats (12, 13). An OT analog (carbetocin) has also been shown to alleviate anxiety, depression, and impaired sociability associated with opioid withdrawal in adult rats (14). Several early stage clinical trials highlight the ability of OT to reduce withdrawal symptoms in alcohol (15) and heroin (16) dependent adults. OT is produced in the hypothalamus, it regulates a complex array of physiological and behavioral processes (17). These neuromodulatory effects of OT have been shown to mitigate the development of addiction and are linked to improvements in stress response and anxiety in both adolescent animal models and children (18, 19). The present study was designed to determine the effect of postnatal OT treatment in rats prenatally exposed to opioids and benzodiazepines. Using a well-established rat model of NAS, we aimed to determine the effect of OT on survival and long-term neurodevelopmental outcomes in rats prenatally exposed to morphine or diazepam.

MATERIALS AND METHODS

Experimental animals - Twenty-two nulliparous timed-pregnant gestational day (GD) two Sprague-Dawley rats (~200g; approximately 70 days of age; Charles River Labs, Hollister, CA) were utilized to establish the NAS models. Another 26 nulliparous timed-pregnant Sprague-Dawley rats were used as drug naïve surrogates for rat pups prenatally exposed to drugs (n=22) and to generate drug-naïve control animals (n=4).

Drugs - Morphine sulfate, diazepam (Sigma Aldrich, St. Louis, MO) and Oxytocin (Phoenix Pharmaceuticals Inc, Burlingame, CA) diluted in in 0.9% saline for subcutaneous (s.c.) injection.

Drug administration and assessment of survival and bodyweight - Beginning on GD2, dams received escalating daily doses of morphine (10-50 mg/kg/day, s.c.) or diazepam (2-15 mg/kg/day, s.c.) until parturition. On the day of parturition (postnatal day [PND] 0), litters from treated dams were cross-fostered to drug-naïve surrogates. All treated dams and drug naïve pups were humanely euthanized. On PND1, each litter (balanced for number of pups) was randomly assigned to OT or saline treatment. In experiment 1, each litter was randomly assigned to receive s.c. injections of either 2 mg/kg/day OT or saline for the first 10 days. Survival and body weight were measured daily using a calibrated precision balance (Mettler Toledo, Columbus, OH). In experiment 2, each litter was randomly assigned to receive s.c. injections of either 0.3, 1, or 2 mg/kg/day OT or saline for the first 10 postnatal days. Survival and body weights were measured daily for 30 days, after which animals were weaned. Surviving animals from experiment 2 underwent behavioral assessments. Blood was collected ~PND46 (see following).

Behavioral assays - Locomotor Assay: On PND 3, 6, 9, and 30, animals were individually placed in a SmartCage™ (AfaSci, Redwood City, CA) for 5 minutes. Distance traveled (cm) and rearing counts were automatically quantified by the SmartCage™ software.

Light-Dark Box Test - A dark box (red transparent enclosure with an opening for entry) was placed in one half of a SmartCage™. Anxiety-like behavior was assessed by measuring the time an animal spends in the "Light Zone" and the number of "Light Zone" entries during a 10 minute period (20).

Passive Avoidance Task- Animals were individually placed into the open chamber of the lightdark box after completely entering the preferred dark chamber a mild foot shock is delivered. Twenty-four hours later, memory for the foot-shock is assessed by measuring the latency to enter the dark chamber during a 5 minute period (21).

Social Interaction Test:- Animals were placed individually in a three-chamber social interaction apparatus attached to a SmartCage™. The test animal was allowed to roam the chamber freely for 5 minutes while both chambers remained empty. Animals that showed a strong preference for either of the two empty social interaction chambers were eliminated. Animals that demonstrated unbiased exploration of each chamber were utilized. A stranger conspecific was placed in one of the empty social interaction chambers and the test animal allowed to explore freely. To determine a preference for social novelty another stranger conspecific was placed in the second social interaction chamber and the amount of time the test animal spent exploring each conspecific was measured over 5 minutes (Social Novelty Test). Social memory was assessed 24 hours later by again placing the animal into the three-chamber social interaction apparatus for 5 minutes and then placing the same two conspecifics in the social interaction chambers and measuring the amount of time the test animal spent investigating each conspecific over 10 minutes (24h Social Memory Test) (20).

Blood collection and Biological Assays - Between PND45 and 47, blood (~2 mL/animal) was collected by terminal cardiac puncture, immediately centrifuged at 5,000 RPM for 5 minutes, and plasma (~1 mL/animal) stored at -70°C. Plasma glucose oxidase levels were measured using an Amplex® Red Glucose/Glucose Oxidase Kit Assay Kit (uM/ml) [Invitrogen, Eugene, OR]. Plasma corticosterone and aldosterone levels were determined using competitive Enzyme Immunoassay kits (pg/ml) [Thermo Fischer Scientific, Inc., Carlsbad, CA]. Plates were analyzed using a microplate reader (SpectraMax M2 with SoftMax Pro 7.1, Molecular Devices, San Jose, CA).

Statistical analysis - Survival was assessed with Kaplan-Meier analyses and the log-rank (Mantel-Cox) test. Two-way analysis of variance (ANOVA) for repeated measures was used for overall comparisons of body weight in experiment 1 and for locomotor activity in experiment 2 with Time (postnatal day) and Treatment (OT and Saline) as factors. One-way ANOVA comparing treatment groups was used for body weight in experiment 2 and 3 and behavioral assessments in experiment 2. Post hoc analyses using Dunnett's, Sidak's, or Tukey's multiple comparison tests were performed. Data was analyzed using Excel and GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA). The significance level for two-sided analyses was set at $P<0.05$.

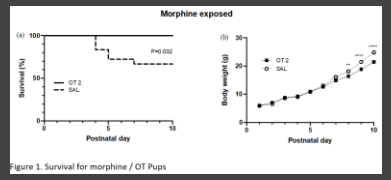

Figure 1. Survival for morphine / OT Pups

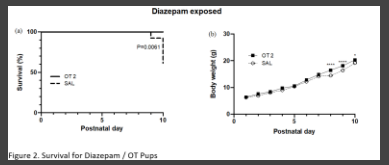

Figure 2. Survival for Diazepam / OT Pups

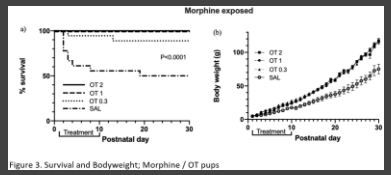

Figure 3. Survival and Bodyweight; Morphine / OT pups

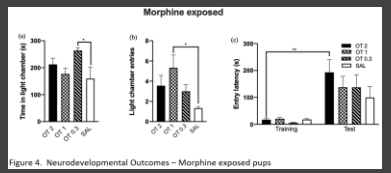

Figure 4. Neurodevelopmental Outcomes – Morphine exposed pups

RESULTS

Experiment 1 - Survival and Body Weight

For pups exposed to morphine, survival significantly improved with OT (χ^2=7.029, P=0.008) (Figure 1a). At PND10, OT survival was 18/18 (100%) compared to 12/18 (66.67%) saline treated animals. There was a significant difference in body weight between the treatment groups at PND8-10, with OT treated animals being significantly lighter (Figure 1b).

For pups exposed to diazepam, OT also significantly improved survival (χ^2=5.924, P=0.0149) (Figure 2a). At PND10, 13/13 (100%) OT treated animals survived compared to 8/13 (61.5%) saline treated animals. Sidak's multiple comparisons test provided evidence for a difference in body weight between treatment groups at PND8 (P <0.0001), PND9 (P<0.0001), and PND10 (P=0.0134) (Figure 2b), with oxytocin treated animals being significantly heavier.

Experiment 2 Survival, Body Weight and Neurodevelopmental measures.

For pups exposed to morphine, OT treatment significantly reduced mortality (χ^2=26.59, P<0.0001). Postnatal treatment with all three doses of oxytocin was associated with improvements in survival (0.3 mg/kg, χ^2=6.66, P=0.0099; 1 mg/kg, χ^2=1.58, P=0.0006; 2 mg/kg, χ^2=1.88, P=0.0006), compared to saline (Figure 3a). At PND30, 16/18 (88.89%) animals treated with 0.3 mg/kg oxytocin and 18/18 (100%) in each group of animals treated with 1 and 2 mg/kg oxytocin had survived, whereas only 9/18 (50%) saline treated animals survived. A significant difference in body weight between OT and saline treated was noted, with oxytocin treated animals being significantly heavier than saline treated animals at PND30 (P's<0.05 to <0.0001; Figure 3b).

There was a significant difference between the treatment groups at PND6 only, with animals treated with 1 and 2mg/kg OT exhibiting greater locomotor activity, compared to saline (Figure 4a). No Treatment main effect for rearing behavior was seen during the Locomotor Assay.

Passive Avoidance Task; There was no difference between treatment groups on test day, but animals treated with 2 mg/kg OT maintained a significant difference between training and test for latency to enter the dark chamber, suggesting that this treatment group was the only group that adequately learned the task (Figure 4d). There were no statistically significant differences between treatment groups on the Social Novelty Test.

Blood corticosterone, aldosterone and glucose oxidase.

There was a significant treatment effect (F(3,57)=5.988, P=0.0013) on glucose oxidase levels, with all OT treatment groups having higher glucose oxidase levels, compared to saline. Plasma corticosterone and aldosterone did not differ between treatment groups (Table 1).

For pups exposed to diazepam prenatally, OT significantly improved survival (χ^2=10.12, P=0.0176; Figure 5a). Follow-up tests revealed that treatment with 1 mg/kg and 2 mg/kg improved survival, compared to saline (Table 1). One dam in the 1 mg/kg treatment group was infanticidal at PND3, all her pups were removed from the analyses. Survival in animals treated with 0.3 mg/kg OT was no different than saline treated animals. At PND30, 8/8 (100%) animals treated with 1 mg/kg OT and 16/16 (100%) animals treated with 2 mg/kg OT had survived compared to 11/16 (68.75%) animals treated with 0.3 mg/kg OT and 10/16 (62.5%) animals treated with saline. There was a significant treatment effect for body weight at PND30 (F(3,41)= 5.018, P=0.0047). Animals treated postnatally with 0.3 and 2, but not 1 mg/kg OT, were significantly lighter than animals treated with saline at PND30 (Figure 5b).

A Time x Treatment interaction (F(9,185)=1.937, P=0.0492) and Time (F(3,185)=76.45, P<0.0001) and Treatment (F(3,185)=11.75, P<0.0001) main effects for distance traveled during the Locomotor Assay were seen. There was a significant difference between animals treated with 0.3 mg/kg OT compared to saline at PND3 and animals treated with 2 mg/kg OT compared to saline at PND 3, 6, and 30 (Figure 6a,b), with the OT treated animals traveling less distance compared to saline treated animals. Once again, a Time x Treatment interaction (F(9,185)=2.843, P=0.0037) and Time (F(3,185)=47.28, P<0.0001) and yand there were no differences between groups at any time point.

Light-Dark Box Test; There was a significant treatment effect for time spent in the light chamber during the (F(3,57)=3.695, P=0.0168). Animals treated with 0.3 mg/kg OT spent significantly more time in the light chamber, compared to saline treated animals (Figure 4b).

Animals treated with 1 mg/kg OT entered the light chamber more often than saline treated animals (Figure 4c).

A Time (F(1,56)=32.91, P<0.0001) effect was seen, but no Treatment main effect or interaction was noted for latency to enter the chamber previously associated with a foot shock in the Treatment (F(3,185)=13.27, P<0.0001) main effects for rearing behavior during the Locomotor Assay were noted. There was evidence for a significant difference between animals treated with 2 mg/kg OT compared to saline at PND3 and between animals treated with 2 mg/kg or 1 mg/kg OT compared to saline at PND30 (Figure 6c,d), with OT treated animals exhibiting less rearing behavior.

A significant treatment effect was seen for amount of time spent in the light-zone during the Light-Dark Box Test (F(3,41)=4.043, P=0.0131). Animals treated with 1 mg/kg and 2 mg/kg OT spent more time in the light chamber compared to saline treated animals (Figure 6e). A statistically significant difference between treatment groups was noted for the number of light chamber entries in the Light-Dark Box Test (F(3,41)=3.991, P=0.0139). Animals treated with 2 mg/kg OT entered the light chamber less often than saline treated animals (Figure 6f).

A Time x Treatment interaction (F(3,56)=3.222, P=0.0293) and Time (F(1,56)=48.61, P<0.0001) and Treatment (F(3,56)=3.393, P=0.024) main effects for latency to enter the chamber previously associated with a foot shock was seen in the Passive Avoidance Task. There was a significant difference between animals treated with 2 mg/kg and 0.3 mg/kg OT, with these animals showing greater entry latencies compared to saline (Figure 6g).

A significant treatment effect (F(3,28)=5.782, P=0.0033) for social novelty on the Social Interaction Test was seen. Animals treated with 2 mg/kg OT spent significantly more time investigating a novel stranger during the Social Novelty Test, compared to saline (Figure 6h).

A significant treatment effect (F(3,41)=3.754, P=0.0180) on glucose oxidase levels was noted. Animals treated postnatally with 0.3 and 2, but not 1 mg/kg OT, had significantly lower glucose oxidase levels compared to saline treated animals. Plasma corticosterone and aldosterone did not differ between treatment groups (Table 1).

Experiment 3.

In control animals born to drug naïve dams, there were no differences in survival between treatment groups, with all animals surviving throughout the experiment. A significant treatment effect for body weight was seen at PND30 (F(3,51)=5.0410, P<0.0039). Animals treated with 0.3 mg/kg OT were significantly lighter compared to saline. There were no differences in body weight between animals treated with 1 or 2 mg/kg oxytocin compared to saline (Table 1).

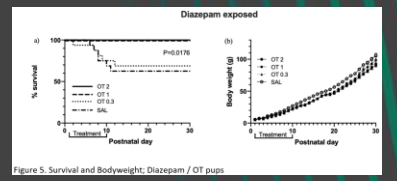

Figure 5. Survival and Bodyweight; Diazepam / OT pups

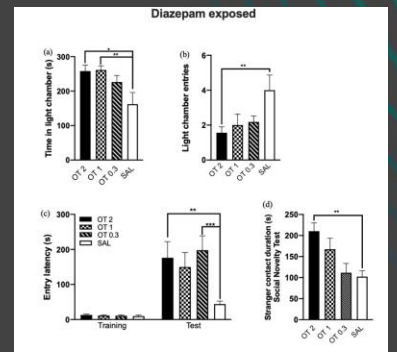

Figure 6. Neurodevelopmental Outcomes – Diazepam exposed pups

DISCUSSION/CONCLUSION

Our data suggests OT treatment significantly improves survival in rats prenatally exposed to morphine or diazepam. OT treatment also positively influences the development of both social and non-social learning and memory processes and reduces behavioral signs of anxiety in animals prenatally exposed to morphine or diazepam. Mortality for human neonates treated for NAS has declined substantially as a result of pharmacological treatment protocols, prenatal exposure to opioids still has the potential to result in death and significant perturbations of development, including delayed growth and behavioral problems (2, 3, 22-24). The mechanisms by which OT improves survival remains unclear, postnatal OT treatment results in improved survival in a genetic mouse model of neonatal hypophagia by inducing feeding (25) and reducing brain injury in a rat model of perinatal asphyxia (26). In the study, pup mortality is restricted to approximately the first 10 postnatal days, suggesting that this phenomenon is driven by acute drug withdrawal.

Although locomotor hyperactivity is widely utilized as measure of acute antagonist-precipitated opioid withdrawal in animal models of NAS (27), our data suggests locomotor hyperactivity is not necessarily exhibited during the later stages of opioid withdrawal since OT had no effect at PND3, 9, or 30. In fact, significantly increased locomotor activity was seen at PND6 in animals prenatally exposed to morphine. In contrast, OT significantly reduced locomotor hyperactivity at PND3, 9, and 30 in animals prenatally exposed to diazepam, highlighting both an acute and long-term effect of OT. The clinical manifestation of benzodiazepine withdrawal are not well understood in the neonate and are further complicated by the dramatically different pharmacokinetic profile of this drug in neonates compared to adults. Although morphine has a similar half-life in human adults and neonates, diazepam has a half-life of approximately 30 hours in adults and up to 31 days in neonates (28). The pharmacokinetic differences of these drugs is evident in the present study when comparing mortality between morphine exposure and diazepam. Clinically, neonates concomitantly exposed to benzodiazepines and opioids are at higher risk of developing severe withdrawal symptoms necessitating pharmacological intervention (29). There are currently no specific treatments for neonatal benzodiazepine withdrawal, so phenobarbital is often used in conjunction with opioids (30). Novel pharmacotherapeutic options that mitigate the use of opioids of phenobarbital which are safe will be of great utility. In addition they may have the potential to mitigate the negative impact of prenatal and postnatal drug exposure on long-term neurodevelopmental outcomes. In the setting of increased metabolic demand, neonates with NAS can experience hypophagia or hyperphagia, but body weight is comparable to the general population during their first year (31). In this study, OT treatment significantly increases body weight in the first 30 postnatal days in animals exposed to morphine prenatally. In contrast, a significant decrease in bodyweight was noted for animals prenatally exposed to diazepam and treated postnatally with OT. Further, glucose oxidase levels followed the same trend as body weight in adolescent animals prenatally exposed to morphine or diazepam with levels higher in animals prenatally exposed to morphine but lower in animals exposed to diazepam. Previous research has shown that postnatal OT increases body weight and blood glucose levels and reduces stress hormones in adult rats exposed to food restriction prenatally (32). In contrast, early treatment with OT has been shown to cause transient inhibition of body weight gain in adolescent rats that recovered after cessation of treatment (18). Clinically, OT has been proposed as a treatment for both hypophagia in infants and hyperphagia and obesity in older pediatric and adult populations (33). It is unclear whether OT has metabolic effects.

Research shows that early treatment with OT improves complex behavioral processes in various species including rats (34), mice (35), monkeys (36), and human infants (33). Results indicated that postnatal treatment with OT reduced signs of anxiety and improvements in memory.

Although a positive trend was observed on most measures, the extent to which OT impacted behavioral outcomes differed depending on the prenatal drug exposure. Without the use of a drug naïve control group for the various behavioral assays, it is challenging to determine if exposure to morphine or diazepam negatively impacted neurodevelopmental functioning. Pre-clinical research highlights OT's modulatory effects across a range of neurochemical processes impacted by drugs of abuse including central monoaminergic activity and control of neural tonicity through regulation of glutamatergic and GABAergic functioning (17). Given the additional evidence that OT reduces signs of acute opioid withdrawal and elevations in cortisol in adult heroin dependent inpatients (16), future research should examine the effect of postnatal OT treatment on stress hormones during acute phase of drug withdrawal in animal models of NAS. In conclusion, we provide the first evidence for a potential therapeutic value of OT in the treatment of NAS. Postnatal OT treatment results in significant improvements in survival and long-term neurodevelopmental outcomes in rats prenatally exposed to opioids and benzodiazepines. If successfully translated into clinical studies, OT has the potential to significantly improve the treatment paradigm for NAS.

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Authorship - DCS, SJA, CP, XSX: Substantial contributions to design, experimentation, data acquisition, analysis and interpretation, manuscript preparation and revisions.

Conflict of Interest Statement - DCS is a founder and major shareholder of Katana Pharmaceuticals Inc. SJA is a shareholder of Katana Pharmaceuticals Inc. CP is an employee of AfaSci, Inc. XSX is a founder and major shareholder of AfaSci, Inc.

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References – Contact Author for full list of citations

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