Differential sensitivity of the Brattleboro rat to glutamatergic and dopaminergic perturbation

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Abstract

Background: The vasopressin deficient Brattleboro (BRAT) rats have behavioral impairments which mimic those seen in other schizophrenia models. However, the mechanism by which vasopressin produces these behavioral abnormalities is unclear. Notably, elevations in dopamine signaling as well as reductions in glutamatergic signaling have been associated with behavioral impairments consistent with those observed in the BRAT rats. Therefore, a potential mechanism for vasopressin induces behavioral abnormalities could be through modulation of dopamine or glutamate signaling. Consequently, the aim of this study was to assess the modulatory function of vasopressin on dopamine and NMDA signaling.

Methods: Single intraperitoneal injection of amphetamine (0.5 mg/kg), a dopamine agonist, and MK801 (0.25 mg/kg), a NMDA antagonist, were used to assess vasopressin (VP) modulatory activity on dopaminergic and glutamatergic pre-pulse inhibition of startle (PPI), social interaction and auditory event related potential (ERPs) impairments in BRAT and littermate control WT rats.

Results: MK801-induced impairments were consistent and not significantly different between WT and BRAT rats suggesting minimal modulatory activity of vasopressin on NMDA signaling. In contrast, amphetamine-induced deficits were genotype dependent. In control animals, amphetamine caused a statistically significant deficit in PPI and social interaction whereas there was no effect in BRAT rats. Conversely, ERP components were unaltered in control rats, whereas N40 amplitude, evoked gamma power, and gamma signal to noise ratio were all elevated in BRAT rats.

Conclusions: The ERP component analyses of BRAT rats treated with amphetamine suggest modulation of auditory information processing through interplay between dopaminergic and vasopressin. However, the behavioral and electrophysiological evidence presented here also suggest that vasopressin does not modulate glutamatergic signaling through NMDA receptors. Further evidence in necessary to determine the interaction between vasopressin and dopamine signaling and future studies are necessary to comprehend glutamatergic interactions with vasopressin that are not NMDA-mediated.

Keywords: Brattleboro, schizophrenia, ERP, PPI, social, vasopressin

Introduction

Schizophrenia is a disabling condition which is characterized by abnormal information processing accompanied with emotional disengagement. These deficits are manifested in conjunction with positive symptoms such as delusions and hallucinations as well as negative symptoms including anhedonia, asociality, and avolition. Although the etiology of schizophrenia is unclear, there is evidence supporting genetic, environmental, and developmental vulnerabilities to disease [1-6]. Current evidence supports two main theories regarding the pathophysiology of schizophrenia, which are the glutamate and dopamine hypotheses [7-9].

The dopamine hypothesis of schizophrenia originated from evidence that excess DA transmission was associated with schizophrenia and inhibition could be used to treat psychosis [10-14]. Further clinical studies provide evidence that the effective dose of antipsychotic drugs is related to their affinity to dopamine type 2 receptors [11-14]. Additionally, neurochemical imaging studies have shown that schizophrenia patients have elevated dopamine receptor availability in certain brain regions [15]. One current interpretation of the dopamine hypothesis is that presynaptic dopamine dysregulation is a common mechanism leading to psychosis in schizophrenia [8]. Alternatively, another review suggests that functional supersensitivity of postsynaptic dopamine signaling is a common mechanism leading to positive symptoms seen in schizophrenia [16]. Although the preponderance of evidence demonstrates a crucial role of abnormal dopamine signaling in schizophrenia, there is also growing evidence for the role other neurotransmitter systems.

The glutamate system has garnered interest because NMDA receptor antagonists such as PCP and ketamine were noted to cause symptoms similar to those seen in patients with various psychological disorders such as schizophrenia [17]. Since the 1950s, the use of NMDA antagonists as a method of modeling schizophrenia in animals has been well established.
The neuropeptide vasopressin (VP) has also been implicated in schizophrenia, as evidenced by the association of a single nucleotide polymorphism in the VP gene with schizophrenia [22]. VP is also involved in behavioral outcomes important in the etiology of schizophrenia such as learning and memory, aggression, and sociality [23]. In addition, both glutamatergic and VP antagonism have been associated with negative symptoms of schizophrenia, suggesting a potential interaction between glutamate and VP signaling in the brain [24-26]. Moreover, there is evidence that VP activity in the hypothalamus may be regulated by glutamatergic signaling [22]. Similarly, VP signaling may also modulate dopaminergic activity as illustrated by an increase in dopamine type 2 receptors in the striatum of VP deficient rats [27-29]. Collectively, the interactive relationship between VP and the dopamine and glutamate systems provide a rationale to consider agents that alter VP activity as potential candidates for pharmacological interventions in schizophrenia.

Methods

Animals

BRAT rats were bred at the University of Pennsylvania from two heterozygous breeding pairs purchased from the Rat Resource & Research Center (RRRC, Columbia, MO). All subsequent rats were bred from heterozygous pairs in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility and housed two to three per cage until electrode implantation and single-housed thereafter in a light- and temperature-controlled room. Light cycle was between 6am-6pm and water and standard rodent chow were available ad libitum. Experiments were conducted in male 300-400g BRAT rats and their littermate controls (WT) at the University of Pennsylvania during the light phase between 9:00 AM and 5:00 PM. Genotypes were determined by sequencing DNA extracted from tail snips for the Avp gene according to protocol provided (RRRC, Columbia, MO). Separate sets of animals were used to perform startle/PPI, social interaction/USV, and electrophysiological testing. All protocols were performed in accordance with University Laboratory Animal Resources guidelines and were approved.
Amphetamine was also dissolved in 0.9% saline to a stock solution of 10 mg/ml and diluted to a 0.25 mg/ml working solution on the day of experimentation. Amphetamine was also dissolved in 0.9% saline to a stock solution of 10 mg/ml and diluted to a 0.5 mg/ml working solution on the day of testing. Rats were weighed prior to testing and drugs were administered in a volume of 1 ml/kg via intraperitoneal injection 15 minutes before tasks.

**Startle and prepulse inhibition**

Startle and prepulse inhibition (PPI) of acoustic startle was assessed (WT n=7, BRAT n=6) using a startle response system (San Diego Instruments, San Diego, CA, USA). Rats were acclimated to the chamber for 5 minutes with background white noise (65 dB) which was also played throughout the test session. Auditory startling stimuli were delivered by a speaker mounted inside the chamber. Startle was detected by a piezoelectric accelerometer mounted beneath the platform. Tones were presented randomly ten times with a randomized inter-trial interval ranging from 10 to 20 seconds. Startle was measured in response to 40-millisecond white noise pulses (tones) between 90–120 dB with 5 dB increments. PPI was done using ten prepulse trials consisted of a 20-millisecond pulse at 69 dB followed by a 40-millisecond pulse of 120 dB with an interstimulus interval (ISI) of 100ms [46]. Prepulse inhibition was calculated as the percent difference in startle responses following the prepulse/startle pair as compared to the startle tone alone.

**Social interaction and ultrasonic vocalizations**

Two previously unpaired rats of the same sex and genotype (WT n=6, BRAT n=6, pairs) were placed in the center of a 32*(W)x18*(D)x12*(H) plastic chamber with no bedding and video was recorded for 5 minutes in low light. Ultrasonic vocalizations (USVs) were recorded simultaneously using an ultrasonic range detector placed 60cm above the chamber (Pettersson Electronik D940 Ultrasound Detector, Uppsala, Sweden). The microphone was interfaced with a Micro1401 data acquisition unit (CED) sampling at 250 kHz recorded through Spike2 software (CED). The chamber was cleaned with 70% ethanol between recordings. The video was manually scored for the amount of time the animals spent interacting. A social interaction was considered to be any time the snout of one animal was in direct contact with the other. Vocalizations were counted manually with the investigator blinded to genotype and drug conditions.

**Event related potentials**

**Electrode implantation**

Animals (WT n=10, BRAT n=10) underwent stereotaxic implantation of three channel electrodes (PlasticsOne Inc., Roanoke, VA) unilaterally placed in the prelimbic cortex (rat prefrontal cortical area) under isoflurane anesthesia, (3.2 mm anterior, 1 mm lateral, and 4 mm deep relative to bregma) and referenced to the cerebellum (2 mm posterior, 2 mm lateral and 2 mm deep, relative to lambda) as previously reported [47-50]. Since the positive and negative electrodes are located far apart from one another, activity recorded using this configuration extends far beyond the localized field generated within the prelimbic cortex, and therefore reflects brain activity across a widespread area similar to a human EEG recording. Histological verification of electrode placement was performed following the completion of experiments. Dental cement and ethyl cyanoacrylate (Elmers, Columbus, OH) were used to secure the electrode pedestal to the skull. Procedures were consistent with descriptions published elsewhere [47,48,51,52].

**Recording**

After a 1 week recovery period, EEG was recorded during the presentation of an auditory task. The auditory task consisted of a single click paradigm with presentation of a 9 kHz tone (10ms, 85 dB) at a 4 second inter-stimulus interval 200 times against 70 dB of background noise. EEG was recorded with a sampling rate of 1667 Hz and band-pass filtered between 1 and 500 Hz during collection. Stimuli were generated by Power 1401 hardware and Spike 6 software (Cambridge Electronic Design, Cambridge, UK) and were delivered through speakers attached to the cage top. All recordings were performed in a plastic cage 7*(W)x11*(D)x5*(H) with standard bedding, which was placed in a Faraday cage 15 minutes before stimulus onset.

**Analysis**

For each animal, individual trials were rejected for movement artifact defined by two times the root mean square of the amplitude per rat. Average waves were created from 1000-ms pre-stimulus to 1000-ms post-stimulus time. The N40 component was defined as the maximum negative deflection between 25 and 60 ms. Power analysis was performed using EEGLAB (Schwartz Center for Computational Neuroscience). Event-related gamma (30-80 Hz and 0-60 ms) power were calculated using Morlet wavelets in 116 logarithmically spaced frequency bins between 4 and 120 Hz, with wavelet cycle numbers ranging from 2 to 10.

**Statistics**

All statistics were performed for each paradigm with the Statistica software package (StatSoft, Inc., Tulsa, OK) using...
a repeated measures analysis of variance (rmANOVA) with genotype as the categorical variable (WT vs. BRAT) and drug condition (saline, amphetamine, MK801) as the repeated variable within each measure. Comparisons were made between WT and BRAT rats following saline and either MK801 or amphetamine, such that each drug was compared to saline in a separate rmANOVA. All post hoc analyses were performed with Fisher Least Squared Differences (LSD).

Results

Startle and PPI
The startle response during saline conditions was elevated in the BRAT rats ($F_{1,11}=9.11, p=0.01$) as previously reported [27]. The administration of MK801 prior to testing did not have an effect on startle response in the WT or BRAT groups ($F_{1,10}=0.45, p=0.52$). Fisher LSD post hoc analyses were explored to assess differential responses between WT and BRAT rats as this was the primary rationale for the study. There was no significant effect of MK801 on either WT (MS=8869.7, df=19.3, $p=0.31$) or BRAT rats (MS=8869.7, df=19.3, $p=0.91$), suggesting that the effect of the drug was similar among both groups. On the other hand, startle was reduced with amphetamine treatment ($F_{1,10}=10.05, p=0.01$). Post hoc analysis indicated that the startle response in the WT rats was not reduced following amphetamine administration (MS=4920.7, df=17.9, $p=0.18$), but the startle response of the BRAT rats was significantly lower following amphetamine than saline (Figure 1A, MS=4920.7, df=17.9, $p=0.01$).

PPI was not different between WT and BRAT rats following saline administration ($F_{1,11}=0.17, p=0.69$), as reported previously [44]. MK801 caused a significant reduction in PPI across both genotypes ($F_{1,11}=10.2, p=0.008$). LSD post hoc analyses were explored to assess differential responses between WT and BRAT rats as this was the primary rationale for the study. There was a significant reduction of MK801 in WT (MS=737.2, df=22.0, $p=0.03$) and BRAT rats (MS=737.2, df=22.0, $p=0.02$), suggesting that the effect of the drug was similar between both groups. Similar to MK801, amphetamine also caused a reduction in PPI ($F_{1,11}=7.44, p=0.02$). In order to determine differential response between the WT and BRAT, post hoc analysis of the genotype by amphetamine interaction indicated a reduction in PPI in WT rats (MS=1561.6, df=19.1, $p=0.01$) but not in BRAT rats (Figure 1B, MS=1561.6, df=19.1, $p=0.4$).

Social interaction
The time spent interacting following saline administration was decreased in the BRAT rats ($F_{1,10}=14.17, p=0.004$) as previously reported [42]. The administration of MK801 did not produce any change in social interaction in the WT and BRAT groups ($F_{1,20}=0.00066, p=0.98$). To assess differential response to MK801 post hoc analyses indicated no change in time spent interaction in WT (MS=250.7, df=20.0, $p=0.79$) or BRAT (MS=250.7, df=20.0, $p=0.82$). Similarly, the administration of amphetamine caused no overall drug effect ($F_{1,19}=1.37, p=0.26$). However, post hoc analysis to assess differential response revealed a significant reduction in social interactions in WT rats following amphetamine (MS=128.4, df=19, $p=0.01$), whereas there was no such difference in the BRAT rats (Figure 2A, MS=128.4, df=19, $p=0.28$).

There was no difference in the number of socially-induced USVs per minute between WT and BRAT rats following saline administration ($F_{1,10}=0.36, p=0.56$). MK801 reduced the number of calls across both genotypes ($F_{1,19}=13.48, p=0.002$). However, there was no difference in the extent to which MK801 reduced the number of calls between genotypes (MS=2189.3, df=20.0, $p=0.04$ for WT, $p=0.006$ for BRAT). On the other hand, amphetamine administration did not produce a main effect across both groups ($F_{1,19}=1.17, p=0.29$). Post hoc analysis revealed a significant increase in USVs among the BRAT rats following amphetamine (MS=4085.5, df=29.0, $p=0.03$) whereas a significant reduction occurred in WT rats (Figure 2B, MS=4085.5, df=29.0, $p=0.03$). This data indicates that during a social context there is a difference in USV
Figure 2. Social interaction and socially induced USVs were measured in freely interacting adult rats. Data indicate that MK801 did not affect social affiliation (A) but amphetamine reduced social affiliation in WT and not BRAT rats. However, socially induced USVs (B) were reduced with MK801 administration in both groups, whereas amphetamine caused a reduction in WT and an increase in BRAT (*p<0.05).

production between WT and BRAT rats with amphetamine treatment but not MK801.

ERPs

There was no difference in N40 amplitude between WT and BRAT rats during saline treatment, however there was a qualitative decrease in amplitude (F₁,₁₈=140, p=0.25). There was a reduction in N40 amplitude with MK801 treatment (F₂,₃₆=16.86, p=0.00001). Post hoc analysis revealed significant reductions in N40 amplitude in both WT (MS=513.5, df=33.3, p=0.006) and BRAT (MS=513.5, df=33.3, p=0.03). Conversely, amphetamine did not produce any statistically significant effect on N40 amplitude across both genotypes examined (F₁,₁₆=0.35, p=0.56). Post hoc analysis of the amphetamine by genotype interaction indicate a significant increase in N40 amplitude in BRAT rats (MS=659.5, df=31.1, p=0.02) whereas there is no difference in response in the WT rats (Figure 3, MS=659.5, df=31.1, p=0.14).

Time frequency measures were calculated in the gamma frequency band (30–80 Hz). Baseline gamma power was not different following saline (F₁,₁₈=1.04, p=0.32). Administration of MK801 caused an increase in baseline gamma power (F₁,₁₈=25.90, p=0.00008) which was consistent in the WT (MS=3.8, df=33.3, p=0.01) and BRAT rats (MS=3.8, df=33.3, p=0.0003). There was no significant effect of amphetamine on baseline gamma power across genotypes (F₁,₁₆=2.39, p=0.14). Post hoc analyses indicated that there was a reduction in baseline power in BRAT rats (MS=6.95, df=35.3, p=0.03), which was not present among WT rats (Figure 4A, MS=6.95, df=35.3, p=0.82).

Evoked gamma power was not different between WT and BRAT rats following saline (F₁,₁₇=0.96, p=0.34). MK801 had no effect on evoked power across both groups (F₁,₁₇=1.51, p=0.24, MS=0.02, df=30.7, p=0.41 for WT, p=0.38 for BRAT). Similarly, there was no effect of amphetamine on evoked gamma power across groups (F₁,₁₆=0.002, p=0.97). However, post hoc analysis of the genotype by amphetamine interaction revealed a trend for a reduction in evoked gamma power in WT (MS=0.02, df=27.2, p=0.09) and an increase in BRAT rats (Figure 4B, MS=0.02, df=27.2, p=0.05).

Gamma SNR was not different among WT and BRAT groups following saline (F₁,₁₇=1.00, p=0.33). The administration of MK801 did not alter gamma SNR (F₁,₁₇=2.05, p=0.17) and no effect was observed in WT (MS=0.0001, df=30.6, p=0.33) and BRAT (MS=0.0001, df=30.6, p=0.32). Likewise, amphetamine administration had no overall effect on gamma SNR (F₁,₁₆=0.37, p=0.55). However, post hoc analysis indicated an increase in gamma SNR in the BRAT rat (MS=0.00001, df=29.7, p=0.02) which was not present among WT rats (Figure 4C, MS=0.00001, df=29.7, p=0.16).

Discussion

The behavioral phenotypes of BRAT rats and other VP deficiency models have previously been studied leading to the idea that VP plays an important role in schizophrenia [27-29, 38-42, 53-58].
Response to NMDA antagonist treatment

The behavioral response to glutamatergic perturbation was assessed using the NMDA antagonist MK801. BRAT rats did not have baseline PPI impairments, consistent with some previously published reports [44,61] but inconsistent with others [29,54,55]. This inconsistency is likely due to differences in the method of PPI measurement. For example, we previously demonstrated that BRAT rats are insensitive to alterations in prepulse intensity, but do display differences in sensitivity to prepulse interval [61]. Consistent with previous studies, MK801 caused a reduction in PPI [62] and caused socially induced USVs (Table 1). However, MK801 failed to produce a change in startle or social interactions. Interestingly, MK801 produced similar effects regardless of genotype on all the behavioral measures tested, suggesting that the behavioral deficits manifested in BRAT rats are not dependent on NMDA signaling. Additionally, MK801 treatment was also used to determine ERP response to glutamatergic challenge in BRAT rats. MK801 treatment induced a reduction in N40 amplitude and an increase in baseline gamma power with no difference found with evoked gamma power and gamma SNR (Table 1). Consistent with behavioral evidence, MK801 induced similar ERP changes in both WT and BRAT rats.

Table 1. Summary of MK801-induced behavioral and ERP effects.

| MK801 | WT | BRAT |
|-------|----|------|
| Behavior | Startle | -- | -- |
| | PPI | ↓ | ↓ |
| | Social | -- | -- |
| | USV | ↓ | ↓ |
| ERP | N40 | ↓ | ↓ |
| | BL | ↑ | ↑ |
| | Evoked | -- | -- |
| | SNR | -- | -- |

The data presented in this study indicate a limited modulatory role on PPI, social interaction, and ERPs between the NMDA system and VP. Previous reports have demonstrated that VP signaling does not alter NMDA or kainate induced HPA activation [63]. However, the experiments done in this study may not have been predictive of the complex interplay between VP and NMDA. For example, others have observed that MK801 administration reduces expression of VP in amygdala and VP analogs ameliorate MK801-induced social

In this study, we intended to clarify some of the potential mechanisms which lead to the behavioral abnormalities relevant to schizophrenia observed in BRAT rats. The two dominant mechanistic theories in schizophrenia posit an increase in dopaminergic tone and a reduction in glutamatergic tone [59,60]. Several studies have shown an exaggerated response to dopamine agonists and NMDA antagonists in patients with schizophrenia. Using pharmacological agents we identified several behaviors and electrophysiological measures that were sensitive to dopaminergic and glutamatergic perturbation in VP deficient BRAT and/or WT rats.

Figure 4. Time frequency analysis of the EEG phase locked to the auditory stimulus is shown for (A) baseline gamma power, (B) evoked gamma power, and (C) gamma signal to noise ratio. MK801 caused an increase in baseline gamma power in WT and BRAT rats. Amphetamine did not change baseline gamma power in WT but was decreased in BRAT rats. Conversely, MK801 had no effect on evoked gamma power and SNR. The BRAT rats demonstrated an elevation in evoked gamma power as well as SNR (†p<0.1, *p<0.05, **p<0.001).
deficits and hyperlocomotion [25,64].

Response to dopamine agonist treatment
The relationship between VP deficiency in BRAT rats and the dopaminergic system was established using the indirect dopamine agonist amphetamine. In contrast to MK801, the effects of amphetamine on the behaviors tested were highly dependent on genotype. In the WT rats, amphetamine-induced behavioral changes included reductions in PPI, social interactions, as well as socially induced USVs. However, there was no difference observed with startle. Conversely in BRAT rats, amphetamine induced a reduction in startle and an increase in socially induced vocalizations. However, there were no differences in amphetamine-induced behavioral changes in BRAT rats on PPI or social interactions. (Table 2). The effects of amphetamine on the behavioral measures examined suggest an interaction between vasopressin and dopamine signaling. Additionally, ERPs were recorded following amphetamine administration in order to assess differential responsiveness in the dopamine system as a function of VP ablation. In line with the effects observed on behavior, amphetamine produced differential effects in the WT and BRAT rats. In WT rats, the amphetamine-induced response was limited to a qualitative reduction in evoked gamma power with no difference seen in the N40 amplitude, baseline gamma power, or gamma SNR. In contrast, amphetamine in BRAT rats produced an elevation in N40 amplitude, a reduction in baseline gamma power, and an increase in gamma SNR, with a qualitative increase in evoked gamma power (Table 2).

Table 2. Summary of amphetamine-induced behavioral and ERP effects.

| Amphetamine-induced behavior | WT | BRAT |
|-----------------------------|----|------|
| Behavior                    |    |      |
| Startle                     | -- | ↓    |
| PPI                         |    | --   |
| Social                      |    | --   |
| USV                         |    | ↓    |
| ERP                         |    |      |
| N40                         | -- | ↑    |
| BL                          |    | --   |
| Evoked                      | -- or ↓ | ↑ |
| SNR                         |    | ↑    |

Interestingly, decreased levels of dopamine and dihydroxyphenylacetic acid (dopamine metabolite) in the frontal cortex have previously been reported in these rats, providing evidence for a reduction in dopamine receptors in the frontal cortex [44], and a possible explanation for the results observed here. Furthermore, D2 receptor binding in the nucleus accumbens shell and dorsomedial caudate are significantly higher in BRAT compared with LE rats [29]. Data in the current study, as well as previously published reports, provide evidence suggesting that BRAT rats are highly sensitive to a dopaminergic perturbation.

Limitations
VP has many important functions including neuromodulation of the HPA axis and the stress response [65]. The BRAT rats have altered HPA axis function that provides an alternative mechanism for the observed phenotypes. Additionally, perinatal and adolescent stressors are risk factors for schizophrenia [66]. Post-mortem studies also indicate that VP and HPA-axis associated constituents such as cortisol are differentially expressed in the pituitary gland of schizophrenia patients [67]. This study did not account for cortisol levels and therefore the effects of the HPA axis alteration were not accessed. Another limitation was the use of single doses for MK801 and amphetamine to assess the effects of NMDA antagonist and dopamine agonist respectively in conjunction with VP deficiency. Nonetheless, the doses used were consistent with those used in rats to elicit deficits in PPI, a consistent endophenotype found in rat models of schizophrenia [68-70]. Furthermore, the doses chosen produced similar increases in locomotor activity, a gross measure of response to the pharmacological intervention. However, since single doses were used in this study it is difficult to make any definitive conclusions on the dose response relationship between dopamine agonist/NMDA antagonist activity and vasopressin deficiency.

Conclusion
Although glutamatergic and dopaminergic signaling are proposed to be involved in schizophrenia, the relationship between vasopressin and these systems has been unclear. We demonstrate through several behavioral and electrophysiological measures that there is limited evidence to support a relationship between VP and NMDA-mediated glutamate signaling. Although there are behavioral abnormalities consistent between hypoglutamatergic function models and VP deficient BRAT rats, these impairments are likely independent of specific deficits in NMDA-mediated glutamate signaling. Conversely, these same measures indicate a significant interaction between VP and dopamine signaling. However, the mechanism through which dopamine and VP interact has yet to be determined. Future studies will address potential regional and molecular mechanisms for the observed differences in sensitivity to alterations of DA signaling.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

| Authors’ contributions | REL | LA | ENB | JDS | MC | AC | VT | REF | SJS |
|-----------------------|-----|----|-----|-----|----|----|----|-----|-----|
| Research concept and design | ✓ |     |     |     |     |     |     |     |     |
| Collection and/or assembly of data | ✓ | ✓ | ✓ | ✓ | ✓ |     |     |     |     |
| Data analysis and interpretation | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |     |     |     |
| Writing the article | ✓ |     |     |     |     |     |     |     |     |
| Critical revision of the article |     |     |     |     |     |     |     |     |     |
| Final approval of article |     |     |     |     |     |     |     |     |     |
| Statistical analysis |     |     |     |     |     | ✓ |     |     |     |
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