Assessment of antidepressant and anxiolytic properties of NK1 antagonists and Substance P in Wistar Kyoto rats

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Abstract

In an attempt to explore the involvement of substance P in depression and anxiety and its’ potential therapeutic effects, we measured basal plasma and hypothalamic levels of substance P in a well-studied animal model of depression — adult male Wistar Kyoto (WKY) rats and their controls, Wistar rats. We also studied the influence of a substance P receptor (NK1) antagonist (SPA) on “anxiety-like” and “depressive-like” behaviors exhibited by the WKY rats in the open field and swim test paradigms, compared to controls. WKY rats exhibited lower levels of substance P compared to controls in the hypothalamus. Though the WKY strain exhibited less rearing behavior in the open field compared to controls, SPA did not influence this pattern of behavior. In contrast, SPA had a significant effect on a depressive-like behavior exhibited by the WKY strain — it reduced significantly the immobility duration of WKY rats in the swim test. Thus it seems that depression involves alterations in levels of substance P, and that NK1 antagonists may be effective in the relief of depressive, but not anxiety symptoms.

Keywords: Substance P; Depression; Anxiety; Rat; Open field; Swim test

Although the monoamine hypothesis is the most dominant attempt to unravel the biological basis of depression, this theory by itself can neither explain the entire mechanism of action of antidepressants, nor can it provide a comprehensive understanding of the pathophysiology of depression [22]. One of the alternative theories relates to the actions of a relatively new class of peptide neurotransmitters known as neurokinins (also sometimes called tachikinins). This approach was generated by observation that an antagonist to one of the neurokinins, namely substance P, may have antidepressant actions [32].

Substance P is released from neurons and preferentially interacts selectively with the neurokinin 1 (NK1) subtype of neurokinin receptor [See review at [55]]. Studies mapping the expression of substance P and NK1 receptors in neural circuits found large concentrations in the amygdala, hypothalamus and hippocampus, areas that are thought to be critical for regulating emotions [52]; See review at [55]).

In most cases, insufficient and/or contradictory evidence exists to establish particular arguments for or against the involvement of substance P in depression and/or anxiety. Several studies demonstrated the antidepressant efficacy of several NK1 receptor antagonists in patients with major depression and high anxiety [32], and in several behavioral measurements in animals [9,14,58,40,48]. According to some researchers, the first genuinely new antidepressant will be a compound that blocks the effect of substance P [23].

On the other hand, there are several studies indicating no or even opposite effects of substance P antagonist on anxiety [33,56,57], for review see [55]) and/or depression [30]. In addition, localized administration of substance P or substance P antagonist in the central nervous system may produce anxiogenic or anxiolytic responses, depending on the animal species and location of injection [15,18,20,21,38,49,56]. Furthermore, to date it is equivocal whether cerebrospinal
fluid (CSF) substance P is altered in depression [19]. Nevertheless, the possibilities of alterations in substance P synthesis and secretion in animal models of depression, and the effects of NK1 receptor antagonists in these models are essentially untested.

In order to establish a particular argument for or against the involvement of substance P in depression and anxiety we used in this study an animal model of depression that exhibits comorbidity with anxiety. This is the Wistar Kyoto (WKY) strain which was derived from Wistar rats. Adults from the WKY strain have been shown to demonstrate depression-like symptoms such as reduced body weight and disturbed REM sleep, compared to Wistar controls [41,43]. They also displayed depression-like symptoms on several behavioral tests, e.g., increased immobility in the swim test and greater degree of “anhedonia” in response to chronic mild stress and acute stress. The increased immobility was prevented by chronic (but not acute) antidepressant treatment [34]. WKY adult rats have been shown to exhibit abnormally low levels of dopamine and its metabolites in the mesolimbic pathway compared to Wistar controls [24,25].

Recently, we reported that prepubertal rats of this strain exhibit increased immobility in the swim test, abnormal social play as observed after 24-h of isolation and increased basal levels of hypothalamic–pituitary–adrenal (HPA) axis hormones [36]. We also found that prepubertal rats from the WKY line showed anhedonia in the saccharin preference test and anxiety behavior in a novel environment [35]. Thus, use of a genetic animal model of depression which exhibits co-morbidity with anxiety, is appropriate and particularly relevant in order to explore the role of substance P in anxiety and depression and the potential therapeutic role of an NK1 antagonist.

In this study, we measured basal levels of substance P in plasma and in the hypothalamus of naïve animals from the “depressed-like” line and their controls. The hypothalamus is a subcortical structure that has a role in depression [60]. It has long been known to mediate many neuroendocrine and neurovegetative functions. Hypothalamic peptides such as corticotrophin releasing factor (CRF), part of the stress responsive hypothalamic–pituitary–adrenal (HPA) axis, and melanin concentrating hormone (MCH) are involved in the regulation of reward and mood [37]. Since the hypothalamus is part of the HPA axis and since the WKY rats exhibited in several studies in our lab [10,36] abnormal levels of HPA hormones, we focused on this subcortical area in this study. In addition, other brain areas also involved in depressive behavior, and where SP is particularly expressed, such as the dorsal raphe, periacqueductal gray, amygdala or PFC should be considered [6,23].

We also used the open field test, which was originally described as a test of emotionality in rats [5]. Anxiety behavior in the open field is triggered by two factors: individual testing (the animal is separated from its social group) and agoraphobia (as the arena is very large relative to the animal’s breeding or natural environment). These two factors may trigger anxiety behavior only in gregarious species and/or in species that show fear of open spaces into which they are forced. This is precisely the case with rodents that live in social group and in small tunnels [45]. In such a situation, rodents spontaneously prefer the periphery of the apparatus to activity in the central parts of the open field, and indeed, mice and rats walk close to the walls, a behavior called thigmotaxis [45]. The open field test studies the animal’s unconditioned or spontaneous behavior, provides a high degree of ecological validity and was extensively validated [46].

In addition we used a paradigm for studying stress responses and screening antidepressant drugs [[1]; but see [29]] — the modified Porsolt [45] forced swim test [39,61,64].

We hypothesized that WKY rats will exhibit abnormal levels of substance P in the plasma and/or in the hypothalamus, and we assumed that if indeed a substance P antagonist has potential therapeutic role on depression and/or anxiety it will improve the behavioral symptoms of the “depressed” line in the open field and/or the swim test compared to it’s influence on the control rats.

1. Method

1.1. Animals

Adult male WKY and Wistar (weight 350–400 g) rats were bred in our colony at Bar-Ilan University. The two strains were likely to be inbred because of the relatively small number of original parents and several generations of in-house breeding. After weaning, juvenile rats were housed in a polycarbonate cage (38 × 21 × 18 cm.), three per cage, in a temperature controlled vivarium (20–24 °C), under 14 h–10 h light:dark cycle (lights on at 0500). Food and water were available ad libitum. The study protocol was approved by the Institutional Animal Care and Use Committee and adhered to the guidelines of the Society for Neuroscience.

1.2. Procedure

1.2.1. Drugs and assays

In the open field and swim test, animals received a dose of 3 mg/kg (a dose found to be effective in earlier studies [16,13]) — of the substance P receptor antagonist (SPA) — [D-Arg¹, D-Phe⁵, D-TRP⁷,⁹, Leu¹¹-NK1 tachykinin receptor antagonist] which was purchased from Sigma (St. Louis, MO) and was described in [16], or saline control. Although the NK1 antagonist, [D-Arg¹, D-Phe⁵, D-TRP⁷,⁹,Leu¹¹] has relatively low affinity for the rat NK1 receptor [27,28], it has been shown that this compound is able to cross the blood–brain barrier (though possibly in low levels [28]) and to reverse the effects of centrally administered substance P in the rat [16].

Intervals between acute injection of SPA and behavioral test varied in different studies between 10–60 min [12–14,17,53,58]. Our procedure included the swim test and the open field test for the same animal — a joint procedure that took between 15–20 min (considering each test was 5 min and the time needed to transfer and prepare the animals and apparatus). In order to test the animals within the 10–60 min post-SPA interval, we chose to wait for 10 min between the injection and the first test (open field).
For the analysis of basal levels of substance P in the plasma and in the hypothalamus we used an Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (Intra assay CV: 4.5%–6.7%, Inter assay CV: 4.2%–7.3%, Sensitivity: 8.0 pg/mL); purchased from MD Biosciences (Zurich, Switzerland; Catalog number DE1400).

1.2.2. Plasma analysis

On the day of the experiment, in each strain, blood samples were taken from naïve males (animals that were not participating in the swim test and open field sessions and were not treated by saline nor SPA). After decapitation, trunk blood was collected into chilled EDTAed tubes. Blood samples were collected after each animal, gloves were changed, and all the equipment was cleaned, in order to prevent pre-decapitation stress. Samples were centrifuged for 10 min at 4 °C at 1000 g, and plasma was stored at −80 °C until determination. On the day of assay, frozen plasma samples were thawed and plasma substance P levels were measured using commercial ELISA kits, as described above.

1.2.3. Hypothalamus analysis

After decapitation, the brains were removed rapidly, and the entire hypothalamus was surgically dissected out with forceps and frozen immediately at −80 °C (as previously described in [62] — see article for definition of anatomical borders of the hypothalamus dissection). Extraction was achieved by thawing the punches and subjecting them to probe sonication (80 W for 5 s with a B-12 Sonifier; Branson, Danbury, CT, USA) in 0.5 ml of a perchlorate solution (0.1 M) containing EDTA/ethanol (0.02:1%) on ice. A sample (100 μl) was removed for protein analysis and the rest was subjected to centrifugation (2000 g, 10 min, 4 °C). The resulting supernatants (the tissue extracts) were filtered (0.45 μm Acrodisk; Gelman, Ann Arbor, MI, USA) and stored at −80 °C until used for the determination of substance P levels by the commercial ELISA kit, as described above.

1.2.4. Open field

The open field arena (62×62 cm) was enclosed by walls 30 cm high. The floor was built from blue plexigal and the walls from green plexigal (a modified version of that used in Janssen et al. [26]). The floor space was divided into 9 equal-size squares (3×3) by a black marker. At the day of the experiment male rats were transferred, separately, to the injection room and placed in cages (identical in size to the home cages) with a thin paper bedding and dried. The cylinder was refilled with fresh paper beddings and dried. The room in which the chamber was located was darkened during testing and the chamber was illuminated by a single 25-W red light bulb, place approximately 50 cm above the base of the chamber. After the open field test (duration of 5 min), animals were moved individually to a clean cage and then to the experimental room and put in the cylinder for a 5 min period. Cumulative time of immobility (defined as no movement of both front paws) was recorded. After 5 min the rats were put in a cage with clean paper beddings and dried. The cylinder was refilled with fresh water after each rat was tested.

Prolonged duration of immobility in this test is often regarded as behavioral despair, an animal analogue of human depression. Inter-rater reliability on the floating measure was =0.948, <p>0.001. N in each group was between 10 and 12.

1.2.5. Swim test

A modified forced swim test that is well accepted in the study of genetic animal models of depression [39,61] was conducted according to a protocol described by [64]. A cylindrical tank (40 cm high, 18 cm in diameter) was filled with water at 3–4 °C above room temperature, to a height (21–24 cm) at which rats could not touch the bottom with their hind paws. The room in which the chamber was located was darkened during testing and the chamber was illuminated by a single 25-W red light bulb, placed approximately 50 cm above the base of the chamber. After the open field test (duration of 5 min), animals were moved individually to a clean cage and then to the experimental room and put in the cylinder for a 5 min period. Cumulative time of immobility (defined as no movement of both front paws) was recorded. After 5 min the rats were put in a cage with clean paper beddings and dried. The cylinder was refilled with fresh water after each rat was tested.

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1.3. Data analysis

Peptide levels in plasma and in the hypothalamus were examined, separately, by t-tests comparing the two strains.

The open field results were analyzed using 2×2 multivariate ANOVA with strain (WKY vs. Wistar) and treatment condition (saline vs. SPA) as the between-subject measures. The dependent measures in the open field were: latency to leave the center square (4) Number of squares entered (“crossing”) (5) Time spent in the corners of the arena (6) (based on Pare [42] — see article for definition of the measures). Inter-rater reliabilities in scoring these behaviors ranged from r=1 to r=0.926, <p>0.001. N in each group was between 10 and 12.

2. Results

2.1. Substance P — plasma levels

As evident from Fig. 1 (right panel), WKY rats tended to have lower levels of substance P compared to Wistar rats, but this was not a significant difference, t(8)=1.872; <p>0.1.
2.2. Substance P—hypothalamus levels

WKY rats exhibited lower levels of substance P compared to Wistar rats \( t(7) = 2.483; p < 0.05 \) (Fig. 1, left panel).

2.3. Open field

2×2 MANOVA revealed a significant effect only for strain \( F(5,38) = 7.03; p < 0.01 \). Examination of the specific differences in each one of the five measures showed a significant difference between the strains in the number of rearing observations \( F(1,42) = 10.26; p < 0.05 \), showing that overall WKY rats (mean: 11.32 ± 2.13) exhibited less rearing compared to Wistar rats (mean: 21.075 ± 2.225) (Table 1).

2.4. Forced swim test

As evident from Fig. 2, in the swim test, significant effects in immobility duration were found, by two-way univariate ANOVA, for strain \( F(1,38) = 51.95; p < 0.01 \) and for strain X treatment condition interaction \( F(1,38) = 4.24; p < 0.05 \). The strain effect showed that, overall, WKY rats exhibited longer immobility duration compared to the Wistar rats. The interaction effect showed that while there were no significant differences between Wistar rats that were treated with SPA or saline, WKY rats treated with SPA exhibited, shorter immobility durations in the swim test compared to WKY rats treated with saline (a significant difference, even though the magnitude of the reduction appears to be small).

3. Discussion

In an attempt to explore the involvement of substance P in depression and anxiety and the potential therapeutic effects of SPA on these mental diseases we measured plasma and hypothalamic levels (one of the brain regions that was found in neuroanatomical studies mapping the expression of substance P and NK1 receptors) of substance P in a well-studied animal model of depression. We also studied the influence of substance P antagonist on the symptoms on anxiety-like and depression-like symptoms exhibited by this strain in the open field and swim test paradigms.

WKY rats exhibited lower levels of substance P compared to their controls in the hypothalamus (and there was a similar tendency in plasma). In addition, although the “depressed-like” strain exhibited less rearing behavior in the open field arena compared to their controls, an anxiety-like behavior, which was found in earlier studies conducted on the WKY strain [27,42], SPA did not modify this behavior. However, SPA had a significant effect on the depressive-like behavior exhibited by the WKY rats — it reduced the immobility duration of the WKY rats in the swim test. We note, however, that in the current study the open field and Porsolt swim tests were conducted one after the other with little time intervals between them. Though we have conducted the less stressful test (open field —

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Saline</th>
<th>SPA</th>
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</thead>
<tbody>
<tr>
<td>Rearing</td>
<td>Wistar</td>
<td>19.25±3.00</td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>12.69±2.88</td>
</tr>
<tr>
<td>Freezing duration</td>
<td>Wistar</td>
<td>13.28±9.07</td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>14.34±8.72</td>
</tr>
<tr>
<td>Latency to leave center</td>
<td>Wistar</td>
<td>2.62±1.21</td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>5.20±1.16</td>
</tr>
<tr>
<td>Crossing</td>
<td>Wistar</td>
<td>40.33±4.47</td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>49.84±4.29</td>
</tr>
<tr>
<td>Time spent in corners of the arena</td>
<td>Wistar</td>
<td>217.22±8.34</td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>205.31±8.01</td>
</tr>
</tbody>
</table>

\( ^1 p < 0.05 \) Wistar vs. WKY.

![Fig. 1](https://example.com/f1.png)

![Fig. 2](https://example.com/f2.png)
emotional stress) before the more severe stressful paradigm (Porsolt swim test — physical stress) in order to minimize the effects of one test on the other [44], possible effects of test order should be considered.

The measurement of CSF substance P concentration in depressed patients is an understudied area, and it is impossible to draw conclusions from the extant, contradictory literature [55]. Rimón et al. [47] reported that depressed patients exhibited a fourfold mean elevation in substance P like immunoreactivity (a finding that was not replicated in another study [8]). A further study [4], reported that lower rather than higher CSF substance P concentrations correlated with psychic anxiety and self-reported symptoms of sadness and inner tension in a group of non-depressed patients, most of whom suffered from chronic pain syndromes. In a study of NK1 receptor autoradiography in cingulate cortex of controls, and patients with schizophrenia, unipolar and bipolar depression, total receptor binding was unchanged, but a decrease in superficial/deep cortical layer NK1 receptor density ratio was noted in the unipolar depression group [11]. The results of our present study support the findings of lower levels of substance P in the CSF of depressed patients. The potential explanation for this finding might emerge from the fact that the WKY strain exhibits symptoms of anxiety, co-morbid with depression [31,35].

From the results of the current study, an apparent discrepancy between the therapeutic efficacy of the neurokinin-1 receptor antagonist and the reduced hypothalamic SP levels might be seen. One should consider that the SP levels from the hypothalamus in this study were from tissue content. These lower SP hypothalamic content levels might reflect either decreased synthesis or increased elimination of SP in the hypothalamus of the WKY rats. In several studies, reduced/increased tissue levels of neurotransmitters/neuromodulators have been taken as an indication of enhanced/poorer neurotransmission respectively [61,63]. Stressful life experience is believed to play a major role in precipitating episodes of many neuropsychiatric disorders including depression [54]. Studies have found that substance P neurons are also responsive to aversive stimuli. Intermittent footshock in rats reduces substance P content in the ventral tegmental area [7], olfactory tubercle [50] and several hypothalamic nuclei [51]. Since the WKY strain shows chronic symptoms of anxiety, the lower levels of substance P in the hypothalamus and in the plasma can be explained by its’ anxiety phenotype.

As mentioned above, the role of substance P in anxiety is controversial. Microinjections of substance P into the rat periaqueductal grey causes both anxiogenesis, as assessed in the elevated plus maze [3] and conditioned place aversion [2]. Conversely, microinjection of substance P into the rat nucleus basalis magnocellularis produces anxiolytic rather than anxiogenic effects in the elevated plus maze and social interaction test [20]. Moreover, the anxiolytic potential of NK1 receptor antagonist is equivocal, regarding evidence from laboratory animals [18,59,65]. The results from our study support the studies that found ineffectiveness of SPA in the relief of anxiety symptoms as exhibited in the open field paradigm by the WKY strain. Nevertheless, one should consider that the negative results reported in this manuscript are interpretable as a lack of effect in anxiety only in the open field paradigm, in which anxiety behavior is triggered, as mentioned above, by unique factors. Other anxiety paradigms, i.e. the elevated plus maze or the novelty suppressed feeding test, which trigger anxiety behaviors by other factors should also be considered.

Though there are some studies contradicting the therapeutic effectiveness of substance P in depression [49], the results from the swim test paradigm in our study support the findings that NK1 receptors antagonists are effective in the relief at least of some symptoms of depression.

In sum, though the complete involvement of substance P in depression is still unclear, from our study it seems that depression involves alterations in plasma and hypothalamic levels of substance P and, that NK1 antagonists are effective in the relief of depressive-like, but not anxiety-like symptoms. Considering the fact that even the newest FDA-approved antidepressant drugs suffer significant drawbacks [55], it seems that there is a place for genuinely new antidepressants that block the effect of substance P.

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