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Lipopolysaccharide-Induced Double Hit on Neurobehaviour and Neurochemistry in the Wistar Kyoto Rat, A Model with Endogenous Depressive-Like Profile

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ABSTRACT

Introduction: The progenitor Wistar Kyoto (WKY) model, outbred from Wistar rats, demonstrates neurochemical and behavioural profiles similar to symptom-presenting depressive patients and may be particularly suitable for teasing out interconnecting phenomena underlying depression and inflammation.

Aims and Objectives: To investigate links between brain function and peripheral infection which has gained currency during the current pandemic, there is a need to find suitable models.

Methods: Lipopolysaccharide (LPS) was administered to assay neurobehaviours, brain and liver enzyme activity. First, baseline indices of anxiety (elevated plus maze-EPM) and learned helplessness (forced swim test-FST) concerning Wistars was obtained.

Results: WKYs demonstrated increased anxiety and inhibitory behaviours in the EPM while risk-taking was reduced. On habituation to the FST, WKY demonstrated reduced immobility, increased swimming and climbing behaviours. In the test, increased adaptive responses were observed. LPS induced a further increase in anxiety levels (EPM) with a concomitant decrease in exploratory behaviour in the novel activity box. Centrally, LPS reduced monoamine oxidase activity in the frontal cortex and hypothalamus while hypothalamic acetylcholinesterase activity was increased. Peripherally, LPS increased liver glutathione activity, with no effect on malondialdehyde at the dose tested.

Conclusion: The WKY model could therefore prove a valuable model to test the 'double hit' hypothesis in understanding the neuro-immune link in depression.

Key Words: Wistar Kyoto rat, Lipopolysaccharide, Depression, Behaviour, Neurochemistry

INTRODUCTION

Investigating links that exist between peripheral infection and inflammation and brain function and dysfunction has become essential because of the currently ongoing pandemic. However, to tease out this circuitry, there is a need to evolve suitable models. The Wistar-Kyoto (WKY) rat, which is an inbred strain of the progenitor Wistar rat arose as a hypotensive control for the spontaneous hypersensitive rat (SHR) model is one such model as it has since been proposed as a putative model of depression. This is because WKYs demonstrated hormonal and physiological measures and depicted behavioural responses similar to those found in symptom-presenting depressive patients. These include, for instance, hyperreactivity to stress, behavioural inhibition, anxiety-like

profile, dysregulation of hypothalamic-pituitary-adrenal (HPA) axis, increased adrenocorticotropic hormone (ACTH) and corticosterone (CORT) levels, neurochemical abnormalities in several systems (dopaminergic, serotonergic and noradrenergic) as well as in peripheral hormones such as thyroid-stimulating hormone (TSH) etc.¹⁻⁶

So the WKY strain, which exhibits endogenous depressive-like behaviour is thought to be impaired in adaptive capabilities, making it more susceptible to exogenous stressors as it demonstrates hypersensitivity to stress with a propensity to develop stress-induced anxiety-like characteristics. However, the WKY strain harbours heterogeneity not found in other inbred strains, including greater behavioural and genetic variability, which has led to mixed results being obtained earlier

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from anxiety and learned helplessness measures with differences emerging between inbred strains of WKY.⁷

Despite this, the WKY rat may be suitable to unravel underlying phenomena that link depression and exogenously-induced stressors or inflammatory states⁸⁻¹¹ as in the case of a diathesis or double hit model, whether through activation of the HPA system or the immune system that lead to altered physiology. Sickness behaviour is an immunological/inflammatory model of depression or a non-specific reaction to various infectious and pro-inflammatory stimuli, such as LPS administration, which constitutes a well-established experimental approach to study the effects of an acute and transient immune activation on physiology and behaviour.⁸⁻¹²

Acting as a stressor,¹² LPS activates common physiological responses (brain cytokine expression, HPA axis activation). How a susceptible WKY strain responds to extraneous immune stimulants would provide a window into understanding vulnerability. LPS elicits a strong immune response leading to the secretion of pro-inflammatory cytokines, which can act on the hypothalamus and other areas of the brain through humoral and nervous routes with profound behavioural deficits like prolonged sleepiness, depression, reduced levels of mobility, anxiety, food and water intake, rearing, grooming.¹³ These complex behavioural changes such as reduced general activity, reduced social motivation and fever response, collectively termed “sickness behaviour,”^{14,12} include reduced exploration, increased anxiety, cognitive dysfunction, and social withdrawal in rodents.¹¹⁻¹⁶ As WKY demonstrate great heterogeneity, it is essential to carry out baseline tests, assessing anxiety- and depressive-like measures. Towards this, the ideal paradigm is the elevated plus maze (EPM), which introduces a conflict between the animal’s inherent urge to explore new environments, vis-à-vis its fear of open, brightly lit spaces. It is, therefore, best suited to assess anxiety-like behaviour. Anxiety and depression often demonstrate comorbidity. Typical features or subtypes of the depression syndrome are better coping style or increased resilience,^{17,18} which are ideally tested out in the forced swim test (FST) in rodents that detects coping strategies in response to stress and is a paradigm for behavioural despair. Immobility or passive behaviour is interpreted as behavioural despair, while swimming is coping with the stressor and climbing is more of defensive behaviour.

Neurochemical and behavioural responses are impacted by LPS administration. LPS induces profound cerebral changes in monoamine metabolism. For instance, at 2-4 hrs post LPS administration, monoaminergic transmission is on the rise with the serotonergic system and the HPA axis is activated.¹⁹ Brain areas such as the frontal cortex are stressor sensitive, while the hypothalamus is the seat of neuro vegetative symptoms associated with sickness behaviour, so we selected these brain areas for assaying ubiquitous acetylcholinesterase (AChE), monoamine oxidase (MAO) and stress marker

neuronal nitric oxide synthase (NOS).

Peripherally, LPS induces inflammation by acting via macrophage TLRs. These activated macrophages initiate a cascade of events culminating in reactive oxygen species (ROS) and generation of free radicals, a reaction that leads to lipid peroxidation, as measured here by liver MDA levels wherein the phospholipid bilayer gets increasingly porous, culminating in necrosis. GSH, the primary non-protein sulfhydryl ubiquitous tripeptide catalyzed by glutamyl cysteinyl synthetase is a potent anti-oxidant scavenging molecule is, which donates its electron to ROS, thus lowering their adverse effect, so liver GSH activity was also measured.

Here, we first established whether the experimental WKY subjects demonstrate anxiety- and depressive-like profiles and then used them for further testing with LPS with behavioural measures being recorded in EPM 2 hours post-injection and a novel activity box 3 hours post-injection. Oxidative stress was quantified by performing biochemical assays of potent anti-oxidant molecules in the liver such as Glutathione (GSH) and levels of malondialdehyde (MDA) were used to assess lipid peroxidation in the liver. Quantification of ubiquitous central acetylcholine degrading enzyme AChE, monoaminergic modulator MAO and gaseous signalling molecule NOS, was carried out. As the immune system is activated in response to mitogens, such as LPS and stressors by inducing an increase in immune cell populations, especially lymphocytes and neutrophils, total and differential leukocyte count [total leukocyte count (TLC), and differential leukocyte count (DLC)] in blood circulation was carried out to detect leukocyte misdistribution. Thus, we screened for anxiety-related behaviours in adult male WKY vis-a-vis Wistar and observed LPS-induced changes in behaviour and neurochemical profiles in WKY.

MATERIALS AND METHODS

Subjects

90-day old, male Wistar (n=8) and WKY (n=8) rats were procured from the ICMR-National Animal Resource Facility for Biomedical Research (NARFBR), Hyderabad, India and housed in the group under standard laboratory conditions with artificial 12h light/dark cycle (lights on at 7:00 h) at an ambient temperature of 22-24°C with free access to food and water. Animals were maintained in groups of 3-4, and experiments carried out according to the guidelines laid down by the Committee for Control and Supervision of Experiments on Animals, Government of India, as per the ARRIVE guidelines and were permitted by Institutional Animal Ethics Committee (No. IAEC/106/2011). All experiments were conducted in the light cycle (9:00–17:00 h). All behavioural recordings were carried out for 5 minutes under 8-8.5 lux as measured at the base of the arena. Recording done with a CCD camera (WV CP500; Panasonic) and data ac-

quisition and analysis done with Ethovision® 9.0 (Noldus, Netherlands).

Baseline behaviour

Wistars (n=8) and WKY (n=8) rats were subjected to baseline testing in the elevated plus maze (EPM), habituated to the swim apparatus and 24hrs later tested in the forced swim test (FST).

Elevated Plus Maze (EPM)

The EPM test was carried out as described in detail elsewhere.^{20,21} Briefly open and closed arm time and entries along with locomotion were automatically quantified. Anxiety index was calculated as open arm time and entries about total time and total entries.²² Non-classical anxiety measures such as nose dips, stretch-attend postures^{22,23} were also quantified.

Forced Swim Test (FST)

The modified FST protocol was adapted^{24, 25} and is a well-characterized paradigm to analyze depression-like behaviour in rodents. The FST was carried out as described in detail elsewhere.^{20,21}

Lipopolysaccharide in WKY rats

24hrs later, WKY rats were randomly divided into two groups. N=4 were injected with 1mg/kg body weight of LPS (*E.coli*, serotype, Sigma) dissolved in saline i.p. with volume made up with saline to 1 ml. Controls (n=4) were injected with the same volume of the vehicle. Two hours after the injection, rats were placed into the EPM as described above.

Activity Box

Three hours after the injection, the rats were placed in the centre of an activity box which was an open cube of 44x44x-44cms. The box was located 50 cms above the ground, and the animal's behaviour was observed for 5 minutes. The observed parameters were ambulation and rearing.

Differential Leucocyte Counting

Immediate after exposure to the activity box, all animals were deeply anaesthetized, and blood collected for mono- and poly-morphonuclear leucocyte (PMNL) staining and quantification. Briefly, blood was smeared and stained with Leishman's stain (Himedia) and observed under a Leica DM2500 and quantification were done using Leica Application Suite software.

Enzyme Assays

Liver and brain tissues of LPS-injected rats (n=4) and control rats (n=4) were taken and deep-frozen until further. Brain areas, frontal cortex and hypothalamus, were dissected out, tissues were homogenized, centrifuged and the supernatant used for spectrophotometric estimations of acetylcholinesterase, nitric oxide synthase, and monoamine oxidase. Liver

tissues were homogenized, centrifuged and the supernatant used for spectrophotometric estimations of Glutathione (GSH), Lipid peroxidation by Malondialdehyde. Enzyme activity was expressed as specific activity in nanomoles or micromoles of the enzyme per mg of protein.²⁶⁻²⁹

Statistical analysis

All data are expressed as group mean \pm S.E.M and tested for statistical differences using t-test. Repeated measures t-test was used to detect differences between habituation and test in Wistar and WKY rats. Differences at p values less than 0.05 were considered significant.

RESULTS

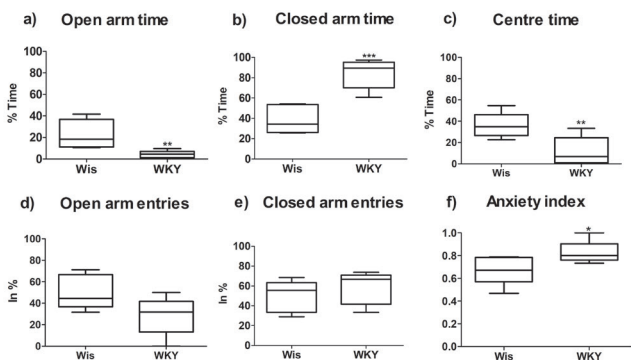
WKYs demonstrated increased anxiety levels with reduced open arm time in the EPM but did not demonstrate learned helplessness in the FST test. LPS induced a decrease in all parameters, notably distance moved and centre time when compared to saline-injected controls, though anxiety-related measures showed no difference. Risk assessment in the EPM and exploratory behaviour in the activity box was significantly reduced. These behavioural changes were accompanied by increased hypothalamic acetylcholinesterase activity and the concomitant decrease in MAO activity in both the frontal cortex and the hypothalamus. Activated brain substrates underlying this were prefrontal and piriform cortices.

EPM

As compared to the parent progenitor strain Wistars, WKYs demonstrated reduced open arm time and increased closed-arm time (fig. 1a,b). Though entries into closed and open arms were not significantly different (fig. 1d,e), the cumulative anxiety-related measures translated into increased anxiety levels in WKYs when compared to age-matched Wistars (fig. 1f). WKYs demonstrated significantly ($t_{df=14} = 4.014$; $p < 0.0017$) reduced ambulation as assessed by distance moved when compared to Wistars. WKYs covered a distance of $9.46 \pm 0.97m$ while Wistars covered close to double the distance at $17.93 \pm 1.66m$. Latency or time taken to enter the open arm was increased in WKYs ($0.32 \pm 0.12s$) as against Wistars ($0.51 \pm 0.20s$), though the difference was not significant ($t_{df=14} = 0.73$; $p > 0.05$). Centre time was significantly reduced in WKY (fig. 1c).

Among the ethological measures, rearing behaviour was comparable between strains (fig. 1g). Risk assessment, as measured in stretch-attend postures wherein the animal while remaining in the confines of the closed arm stretches into the open arm, were significantly increased in WKY (fig. 1i). Head dips, indicative of risk-taking, wherein the animal positioned in the open arm dips its head, were significantly reduced in WKY (fig. 1h). For Mean \pm SE values of anxiety-related and ethological measures, see fig. 1.

Anxiety-related Parameters



Ethological Parameters

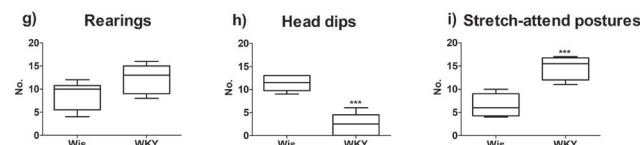


Figure 1: Anxiety-related and ethological parameters in the EPM of WKYs vs. Wistars. Top panel: Duration spent in the a) open arm ($t_{df=14}=3.35$; $p=0.0074$) was significantly reduced in WKYs with a corresponding increase in b) closed arm time ($t_{df=14}=5.84$; $p=0.0002$), while c) centre time was also significantly reduced in WKYs ($t_{df=14}=3.776$; $p=0.0026$). Middle panel: Entries into the d) open arm ($t_{df=14}=2.412$; $p=0.05$) were significantly reduced in WKYs while those into the e) closed arm were comparable with Wistars ($t_{df=14}=0.7699$; $p=0.4592$). Anxiety index was significantly higher in WKYs than Wistars ($t_{df=14}=2.636$; $p=0.0249$). Lower panel: Ethological parameters: a) Rearing demonstrated a trend ($t_{df=14}=2.073$; $p=0.0625$) while stretch-attend postures ($t_{df=14}=5.302$; $p=0.0003$) were significantly increased in WKY. Head dips from the open arm were significantly reduced ($t_{df=14}=7.571$; $p<0.0001$) in WKYs. Whiskers represent minimum to maximum (variability in the samples); box represents occurrence of majority of the samples (75%; 25%), midline is the median.

FST

During the habituation exposure, WKY spent $25.05 \pm 3.66\%$ of the time in the FST immobile while Wistars were immobile for $36.74 \pm 1.88\%$, the strain difference being significant ($t_{df=14}=3.091$; $p=0.0112$). WKY demonstrated increased mobility or swimming in the FST with $28.79 \pm 2.32\%$, when compared to Wistar who spent $22.15 \pm 1.41\%$ of the time swimming, the difference being significant ($t_{df=14}=2.57$; $p=0.0245$). WKY demonstrated increased climbing behaviour in the FST with $42.03 \pm 3.47\%$ time spent in climbing, when compared to Wistar who spent $38.15 \pm 2.04\%$ time in climbing, the time being comparable ($t_{df=14}=0.999$; $p>0.05$). No differences were observed in latency (time taken) to immobility (Wistar: 43.74 ± 5.54 ; WKY 49.80 ± 7.05 ; ($t_{df=14}=0.6848$; $p>0.05$).

During the test exposure, WKY spent $9.08 \pm 1.75\%$ of the time in the FST immobile while Wistars spent $21.69 \pm 3.92\%$ of the

time immobile, the difference being significant ($t_{df=14}=2.560$; $p=0.0284$). WKY demonstrated increased swimming behaviour in the FST with $22.52 \pm 0.89\%$, when compared to Wistar who spent $20.08 \pm 1.11\%$ time in swimming, though the difference was not significant ($t_{df=14}=1.63$; $p>0.05$). WKY demonstrated increased climbing behaviour at $64.70 \pm 4.10\%$, when compared to Wistar who spent $51.82 \pm 4.96\%$ time in climbing, the difference not being significant ($t_{df=14}=1.957$; $p=0.07$). No differences were observed in latency to immobility (Wistar: 26.71 ± 5.78 ; WKY 31.73 ± 8.226 ; ($t_{df=14}=0.5105$; $p>0.05$).

Hab vs Test

Differences between habituation and test are depicted in fig. 2. WKY took a lesser time to become immobile (fig. 2a), demonstrated significantly reduced immobility (fig. 2b) and increased climbing (fig. 2d) behaviour during the test. Swimming behaviour was comparable between habituation and test. Wistars, on the other hand, also demonstrated significantly reduced immobility (fig. 2b) and increased climbing (fig. 2d) behaviour during the test. Their latency to immobility (fig. 2a) and swimming behaviour (fig. 2c) were comparable between the two exposures. The repeated measures t statistic and p values are depicted in fig. 2.

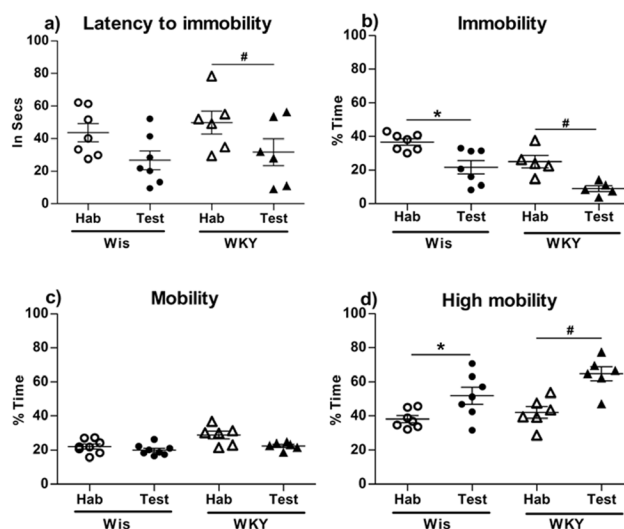


Figure 2: Forced Swim Test. Differences between habituation and test. a) Latency/time taken to Immobility/become immobile: Wistar - $t_{df=7}=1.231$; $p=0.273$; WKY - $t_{df=7}=2.645$; $p=0.0457$; b) Immobility: Wistar - $t_{df=7}=3.872$; $p=0.0117$; WKY - $t_{df=7}=3.331$; $p=0.0447$; c) Mobility/Swimming behaviour: Wistar - $t_{df=7}=1.291$; $p=0.2378$; WKY - $t_{df=7}=2.282$; $p=0.0714$; d) High mobility/Climbing behaviour - Wistar - $t_{df=7}=2.652$; $p=0.0380$; WKY) - $t_{df=7}=3.940$; $p=0.0110$.

LPS in WKY - EPM

LPS treatment reduced locomotory activity by inducing a significant ($t_{df=6}=3.344$; $p=0.0086$) reduction in distance moved (Veh: 1.14 ± 0.10 m vs. LPS: 0.59 ± 0.13 m). Latency to enter the open arm was comparable, with Vehicle-treated animals

taking 40.97 ± 19.36 s while LPS-treated rats took 22.03 ± 12.87 s to enter the open arm ($t_{df=6} = 0.8144$; $p > 0.05$). Entries into the open arm were very few (Veh: 0.16 ± 0.02 s vs. LPS 0.14 ± 0.08 s; $t_{df=6} = 0.220$; $p > 0.05$) as also closed arm entries (Veh: 0.86 ± 0.33 s vs. LPS 0.91 ± 0.06 s; $t_{df=6} = 0.902$; $p > 0.05$).

LPS affected center time which was significantly ($t_{df=6} = 3.59$; $p = 0.007$) reduced in LPS-treated rats (10.17 ± 2.86 s) as compared to controls (34.75 ± 7.37 s). Open arm time was significantly reduced in LPS-treated rats (Veh: 4.20 ± 1.18 ; LPS 0.70 ± 0.47 ; $t_{df=6} = 2.305$; $p = 0.05$). Closed arm time was significantly ($t_{df=6} = 3.752$; $p = 0.005$) higher in LPS-treated rats (287.2 ± 3.85 s vs. Veh: 259.8 ± 6.98 s). For % open arm time, % centre time and % closed arm time see fig. 3a.

LPS induced a decrease in the ethological parameters tested such as rearing frequency (Veh: 8.00 ± 0.55 ; LPS: 4.60 ± 1.29) which were significantly reduced ($t_{df=6} = 2.429$, $p = 0.041$), as also stretch-attend postures from the safety of the closed arm onto the open arm (Veh: 8.60 ± 1.17 ; LPS: 3.80 ± 1.16 ($t_{df=6} = 2.921$, $p = 0.019$).

LPS in WKY - Activity box

3 hours LPS injection, reduced ambulation was observed, with vehicle-treated WKY demonstrating 8.78 ± 0.56 m while LPS-treated rats demonstrated reduced locomotor activity at 3.59 ± 0.60 m moved; $t_{df=6} = 6.284$; $p < 0.0001$). Rearing behaviour was also influenced by LPS: vehicle-treated rats exhibited 7.18 ± 0.91 rears vs. LPS-treated rats which exhibited 3.20 ± 1.16 rears, the difference being significant ($t_{df=6} = 2.735$; $p = 0.023$). There was no significant difference in centre duration with vehicle-treated rats depicting 2.90 ± 0.98 s in the center of the activity box vs. LPS-treated rats which demonstrated 38.30 ± 30.64 s, the difference being not significant ($t_{df=6} = 1.155$; $p = 0.2750$). Neither was there any difference in time spent in the periphery (Veh: 297.3 ± 0.98 s vs. LPS: 261.9 ± 30.64 s; $t_{df=6} = 1.155$; $p > 0.05$). For activity

box measures of vehicle vs. LPS treated animals three hours following the LPS injection, see fig. 3b.

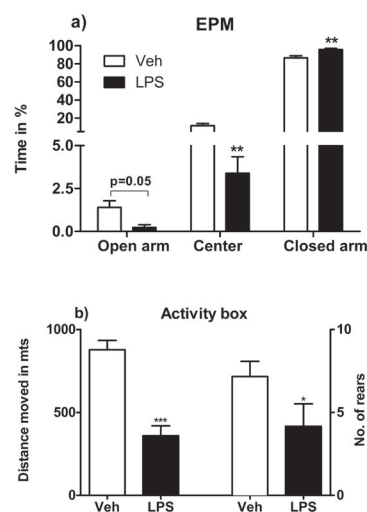


Figure 3: a) In the EPM at 2 hrs post LPS injection, LPS-induced effects on open arm, centre and closed arm times. There was a significant reduction in centre time of LPS group ($t_{df=6}=3.59$; $p=0.007$), a significant increase in closed arm time ($t_{df=6}=3.752$; $p=0.005$) while open arm time just reached significance ($t_{df=6}=2.305$; $p=0.05$). For time in secs, see text. b) At 3hrs post LPS, distance moved and rearing behavior in the activity box. Distance moved: Veh: 8.783 ± 0.5639 m; LPS: 3.593 ± 0.6035 ($t_{df=6}=6.284$; $p < 0.001$). Rearing behaviour: Veh: 7.167 ± 0.91 ; LPS 3.20 ± 1.16 ($t_{df=6}=2.735$; $p=0.023$).

PMNL vs MNL

Differential leucocyte count in vehicle and LPS-treated groups demonstrated a decrease in Lymphocyte and monocyte counts in LPS-treated animals. The decrease in the lymphocytes ($t_{df=6} = 6.693$; $p < 0.001$) and monocytes ($t_{df=6} = 57.00$; $p = 0.002$) was very significant. LPS treatment induced a significant rise in neutrophils ($t_{df=6} = -10.220$; $p < 0.001$).

Table 1: LPS induced an increase in cholinergic function, while both MAO and NOS enzyme activity was reduced on LPS treatment. – Hypothalamus – Frontal cortex (FrA and PFC)

Organ	Enzyme/Product	Region	Vehicle	LPS	t statistic; p value
Brain	AChE nmoles/mg	FC	0.067 ± 0.005	0.069 ± 0.004	$t_{df=6}=0.329$; $p=0.74$
		Hypothal.	0.075 ± 0.004	0.087 ± 0.002 ↑	$t_{df=6}=2.466$; $p=0.03$
	NOS nmoles x 10^{-5} /mg	FC	6.58 ± 0.58	6.14 ± 0.50	$t_{df=6}=0.570$; $p=0.58$
		Hypothal.	7.13 ± 0.42	6.57 ± 0.24	$t_{df=6}=1.204$; $p=0.25$
Liver	MAO μ moles x 10^{-5} /mg	FC	3.70 ± 0.57	1.94 ± 0.31 ↓	$t_{df=6}=2.865$; $p=0.02$
		Hypothal.	4.16 ± 0.47	2.34 ± 0.53 ↓	$t_{df=6}=2.569$; $p=0.03$
	GSH μ g/mg		14.11 ± 0.65	16.59 ± 0.53 ↑	$t_{df=6}=2.946$; $p=0.01$
		MDA μ g/mg		9.40 ± 0.77	8.61 ± 0.74

Liver and Brain Enzyme Assays

In the brain tissues, a significant increase in AChE levels was observed in the hypothalamus in LPS-treated rats, but no difference was observed in the frontal cortex. NOS levels were reduced on LPS treatment in both frontal cortex and hypothalamic regions, though group differences were not significant. MAO was significantly reduced in both the frontal cortex and hypothalamus (Table 1).

The liver tissues of LPS-treated rats demonstrated a significant increase in GSH activity when compared with vehicle-treated controls. Malondialdehyde levels were not affected by the LPS injections and remained at comparable levels in both treated and control groups. The effects of LPS on AChE, MAO and NOS enzyme activity in brain and liver antioxidant enzyme activity and lipid peroxidation are summarized in Table 1.

DISCUSSION

It was essential to establish an anxiety- and depressive-like profile in WKY before using the WKY for the further challenge using LPS. Elevated plus-maze was used to screen for anxiety levels and risk assessment behaviours. WKY depicted increased anxiety-like behaviour in the EPM when compared to age-matched Wistars which was evident from the reduced time spent and reduced number of entries into aversive areas. These together with increased closed-arm time and despite comparable closed arm entries translated into increased anxiety in WKY as compared to age-matched Wistars. The number of closed arm entries which is the best measure of locomotor activity,²⁸ was comparable indicating that the other behaviours were not confounded by a difference in locomotion.

Though the EPM is conventionally used to assess anxiety-like behaviour,³⁰ it also enables assessment of some other complementary, ethological parameters that offer insights into behavioural and physiological effects in rodents, such as risk-taking wide head/nose dips, risk assessment wide stretch-attend postures etc.^{31,32} The latter indicates that the animal is hesitant to move from its present location to a new position and thus a high frequency of these postures as was observed in the WKY indicates a higher level of anxiety. The reduced head/nose dips, indicative of reduced risk-taking, arose as a result of the reduced open arm entries.

In the FST, used to assess depressive-like behaviour, WKY demonstrated reduced immobility when compared to Wistars, though both strains did not demonstrate learned helplessness, the construct of the FST. This is at variance with another study on adult WKY study which showed increased immobility as indicative of helplessness with a propensity to develop stress-induced anxiety-like characteristics.³³ Moreover, both strains demonstrated similar variations between the

habituation and test exposures, with reduced immobility and increased struggling behaviour manifested in the test, though others suggest that strain-specific behavioural differences emerge between habituation and test.³⁴ Here, sample size may have been short to bring out strain-specific differences, particularly in life-death situations, where both strains demonstrated comparable coping strategies as in swimming and comparable struggling as a defensive behaviour.

The similarities could be due to a modified protocol being used, or the size of the FST apparatus. It has also been suggested that the FST does not mimic the causal or phenomenological features of major depression³⁵ and that FST measures define behavioural depression only operationally. Differences also may be less marked when WKYs are compared to a single strain (Wistars) as here, or other strains such as SD, SHR, FSL.^{1,36-39} The WKY rats may have been sensitized as latency to immobility is reduced during the test or may have been able to adapt to the aversive situation by demonstrating increased struggling or defensive behaviour. Better coping style or increased resilience is also considered typical features or subtypes of depression syndrome.^{17,18}

Mixed results have been obtained earlier from the EPM and the FST in WKY, with differences in immobility between inbred strains of WKY.⁷ However, due to more striking neurophysiological characteristics, than those tested here, the WKY has been proposed as a model with endogenous anxiety- and depressive-like features that can be used to test out the double-hit hypothesis, wherein genetic predisposition combined with subsequent environmental insult can precipitate neural psychopathology.

LPS induced a decrease in all parameters in the EPM, notably open arm time, distance moved, centre time, rearing frequency and stretch-attend postures when compared to saline-injected controls, while closed-arm time was concomitantly increased. At 2 hrs post LPS administration, many symptoms of sickness behaviour are said to reach their plateau as central monoaminergic activity is reduced. Two hours post LPS administration, the behavioural response could demonstrate coping strategy in the form of attempts made by the organism to tide over the inflammation and cope with its effects on various physiological pathways, both at the level of the brain and in the periphery. When LPS-administered rats were introduced into the EPM 3 hrs post-injection, entries into the open arm were reduced, as also time spent in the open arm.⁴⁰

The activity box provided simultaneous measures of locomotion, exploration and anxiety 3hrs post-LPS administration. Again, distance moved and rearing frequency, which are measures of socially motivated exploratory behaviour was significantly reduced in LPS-treated rats. Similar responses that are reduced ambulation and rearing persist also at 4 hrs post LPS injection.⁴⁰ Other studies have observed no behavioural changes occurring between 1-3 hours after injection.¹²

Importantly for this study, pain facilitation or hyperalgesia, viewed as an integral part of sickness behaviour, aimed at restricting activity^{41,42} may have been manifested. Locomotor activity is a DA-mediated behaviour with LPS inducing a modest increase in DOPAC thus lowering DA.⁴³ The behavioural changes reflect sickness behaviour as evidenced by reduced exploration and increased anxiety.

Such sickness behaviour, which includes retardation of motor activity, reduced interest in exploration, decrease in social activities accompanied by loss of interest or pleasure (anhedonia), and impaired cognitive function,³⁸ are typical of anxiety- and depressive-like profiles in WKY^{20,21,43,6} with similar behaviours manifesting on acute administration of LPS in rats.⁴⁴ Therefore, the WKY seemed ideally suited to test out the double-hit hypothesis of anxiety-related behaviour, demonstrating that immune activation produces an enhanced propensity toward anxiety-related behaviour and that this susceptibility is associated with alterations to the HPA axis as we have shown earlier.²¹ In fact, it has been observed⁴⁵ that neonatal LPS induced anxiety-like behaviours in adult rats. The altered neural substrate on LPS administration is said to be enhanced serotonergic function.¹⁹

LPS induces inflammatory cascades resulting in sickness behaviour, as peripheral LPS administration results in increased plasma and central levels of multiple proinflammatory cytokines in a time-dependent manner⁹. 6 hours post LPS injection there was an increase in brain TNF α , IL-1 β and IL6 cytokines.⁴⁰ Peripherally in plasma, there is an increase in the PMNL, whereas the decrease in the MMNL in the LPS-administered rats. It is assumed that the decrease in the lymphocytes may be due to LPS activation with stress altering total and differential leukocyte count in blood circulation thereby inducing leucocyte misdistribution.

LPS is a stressor¹² that activates common physiological responses (brain cytokines expression, HPA axis activation) as a stressor would, in the case of WKY it would again substantiate the double-hit hypothesis. A peripheral inflammation had an effect on central hypothalamic AChE with the increase in AChE activity, thus indicating increased hydrolysis of Acetylcholine (ACh) in cholinergic synapses, an increase indicating increased cholinergic signalling or increased recycling, such that reduced ACh is there in the synaptic cleft.^{46,47} However, it was not affected in the frontal cortex.

MAO was reduced in both the frontal cortex and hypothalamus following LPS administration. WKYs have been shown to demonstrate reduced hypothalamic serotonergic activity, particularly in males.¹⁹ On exposure to stress, serotonin (5-HT) levels in another brain that is Nucleus Accumbens (shell region) of the striatum were found to be reduced.⁴⁸ Similar dysregulation of serotonergic neurotransmission or signalling underlies negative mood aspects of MDD associated with vulnerability to developing mood disorders.

A peripheral inflammation, such as LPS administration can affect central ubiquitous enzyme so Neuronal nitric oxide synthase (nNOS), which is the constitutive, neuronal form of NOS and is an indicator of nitric oxide(NO) release in response to inflammatory-related actions in neuronal tissue and is found in 2% of neurons spread across the brain was quantified 3 hours post LPS administration. LPS did not affect nNOS activity either in the frontal cortex or in the hypothalamus, indicating no effect at the dosage used. Overproduction of NO in inflammation is due to inducible Nitric Oxide Synthase (iNOS) which is upregulated in response to LPS, but this is not observed in brain tissue.

It is as yet unclear as to whether LPS can cross the blood brain barrier, or mediates its effects via the vagus nerve or just by inducing release of surrogate molecules that has an effect centrally. Peripherally, it activates the innate immune system via TLRs and intracellular NF- κ B signaling. LPS given in multiple doses over 2-4 days is said to activate resident microglial cells though pathways remain to be determined. It does involve cytokines such as TNF- α , or endothelial cell TLR-mediated production of cytokines⁴⁹ that activate resident microglia. It is suspected that these microglia perform neuroprotective functions.⁵⁰ LPS can act via immune activation-mediated release of pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-1, IL-6 which can pass through the BBB and act on the brain.

LPS induced oxidative stress and effect on lipid peroxidation as assayed in liver tissue demonstrated that LPS induced a significant increase in non-enzymatic antioxidant GSH levels while MDA levels as indicative of lipid peroxidation were not affected at the dose that was tested. Non-enzymatic GSH is a major endogenous antioxidant that modulates redox-regulated signal transduction, apoptosis and cell proliferation. It decreases ROS such as H₂O₂, superoxide anion, singlet oxygen and hydroxyl radicals or acts as a substrate to GSH peroxidase catalyzing elimination of H₂O₂, lipid peroxides, peroxy nitrite etc. Prolonged generation of ROS can eventually lead to lipid peroxidation, wherein the phospholipid bilayer gets increasingly porous, culminating in necrosis. GSH has been shown to significantly decrease LPS-induced inflammation⁵¹ and is probably the reason why no effect on lipid peroxidation was observed here.

Symptoms of sickness behaviour, major depression or even clinical depression overlap considerably.⁴¹ Clinical depression associated with various medical conditions is not merely a reaction that accompanies the physical disease process but may be directly caused by activation of the immune system.⁸ Following LPS administration, subjects demonstrate a transient substantial increase in levels of anxiety and depressed mood. Beyond the recognized role of monoaminergic systems in the pathophysiology of depression, a strong link between depression and inflammatory phenomena has

emerged with brain cytokines (IL-1 β) influencing the neurochemical systems involved in depression. Indeed, it has been hypothesized that depressed patients may be suffering from a dysregulation of the immune system with increased production of pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α .⁹ Moreover, the administration of cytokines is well known to induce unfavourable symptoms which have many features in common with major depression.

Systemic LPS injection to animals causes effects comparable to those found in humans. The findings in WKY rats support the hypothesis that immune activation is involved in the cause and symptoms of illness-associated depression.¹⁰ The inflammatory mediators or cytokines then not only control local and systemic immune responses but also exert effects on the central nervous system with a key role played by brain cytokines in mediating the observed behavioural changes. LPS induced neuronal activity in specific brain areas could also lead to pathologies as neurochemical systems involved in depression were also involved.

CONCLUSION

The findings of this study indicate that the WKY with endogenous depressive-like profile could serve as a suitable model to unravel underlying phenomena in conditions of induced stress in vulnerability leading to double-hit like pathological conditions.

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