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ORIGINAL RESEARCH

Sildenafil, alone and in combination with imipramine, displays antidepressant-like effects in a time-dependent sensitisation-induced (TDS) rat model of treatment-resistant depression

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Background: Despite numerous pharmacotherapies at our disposal to treat major depressive disorder (MDD), extremely high rates of treatment resistance prevail, while burdensome side effects are common. To address these challenges and improve treatment outcomes, there is an urgent need for novel, improved and better-tolerated pharmacological options. Evidence suggests that targeting mood-modulating biochemical systems other than the monoaminergic system is essential, offering promising alternatives. In this regard, pathophysiological features of MDD, including hypothalamic-pituitary-adrenal (HPA) axis hyperactivity, impaired neuroplasticity, neuroinflammation, immune dysfunction and oxidative stress, represent targets of interest. The selective phosphodiesterase type 5 (PDE5) inhibitor, sildenafil, is one such potential candidate, with pre-clinical data suggesting it has antidepressant-like properties. Although speculative, these actions may involve altered neuroplasticity, and anti-inflammatory and antioxidant actions. However, its therapeutic potential in treatment resistant depression (TRD) has not been explored.

Aim: To investigate whether sildenafil, either as a monotherapy or as augmentation with a traditional antidepressant, can exert antidepressant-like bio-behavioural effects in a rodent model of TRD, and whether such actions involve the above-mentioned processes.

Methods: A time-dependent sensitisation-induced (TDS-induced) model of TRD in Flinders Sensitive Line (FSL) male rats was used. TDS-naïve groups were left undisturbed from postnatal day 40 (PND40) to PND54, whereas TDS-exposed groups were subjected to a TDS paradigm from postnatal day PND40. Starting on PND55, rats received either vehicle control (VEH), imipramine (15 mg/kg/day) (IMI-15), sildenafil (3 mg/kg/day) (SIL-3), sildenafil (10 mg/kg/day) (SIL-10) or IMI-15 + SIL-3 for 10 days. The open field test (OFT) and forced swim test (FST) were performed on PND62 and PND63, respectively. Hippocampal brain-derived neurotrophic factor (BDNF), serotonin (5-HT), norepinephrine (NE), glutathione (GSH), and glutathione disulphide (GSSG) levels were assayed on PND65.

Results: TDS exposure elevated immobility time in FSL rats (depressive-like behaviour) in the FST (p = 0,0186) compared to stress-naïve FSL rats. Sub-chronic IMI-15 treatment decreased immobility in TDS- compared to VEH-treated TDS-exposed rats (p = 0,0097), hence inducing a treatment response in TDS rats. SIL-10 (p = 0,0196), and IMI-15 + SIL-3 (p = 0,0005) were similarly effective while SIL-3 was not. Importantly, SIL-3 augmented the antidepressant-like effects of IMI-15, with IMI-15 + SIL-3 displaying greater immobility-reducing capability compared to IMI-15 in TDS-exposed rats. Moreover, TDS exposure elevated hippocampal BDNF (p = 0,0007), 5-HT (p < 0,0001), NE (p = 0,0227), GSH (p < 0,0001), and GSSG (p = 0,0185) levels in FSL rats compared to non-stressed FSL rats. IMI-15 (p = 0,0317) and SIL-10 (p = 0,0078) reduced hippocampal BDNF levels in TDS-exposed rats but did not influence hippocampal 5-HT, NE, GSH, and GSSG levels compared to VEH. In addition, compared to VEH, sub-chronic SIL-3 treatment reduced hippocampal GSH (p = 0,0018) levels of TDS-exposed rats without altering hippocampal BDNF, 5-HT, NE, and GSSG levels. Sub-chronic IMI-15 + SIL-3 treatment reduced hippocampal BDNF (p = 0,0024), 5-HT (p = 0,0115), GSH (p < 0,0001), and GSSG (p = 0,0002) levels of TDS-exposed rats compared to VEH, but without affecting hippocampal NE levels. SIL-3 augmented the 5-HT- (p = 0,0115) and GSSG-lowering (p = 0,0002) effects of IMI-15, with IMI-15 + SIL-3 reaching statistical significance in TDS-exposed rats but not IMI-15 or SIL-3 alone. Finally, SIL-3 augmented the GSH-reducing action of IMI-15 (p = < 0,0001) compared to SIL-3 alone (p = 0,0018), whereas IMI-15 by itself had no such effects in TDS-exposed rats.

Conclusion: TDS + FSL rats displayed depressive-like behaviour and evidence for oxidative stress but paradoxically with elevated hippocampal monoamines and BDNF. This apparent counterregulatory action of BDNF is reaffirmed by IMI-15, SIL-10, and IMI-15 + SIL-3 reversing elevated BDNF and depressive-like behaviour in TDS + FSL rats. Also, SIL-3 only reversed some redox changes, whereas IMI-15 + SIL-3 reversed serotonergic and all redox changes. SIL-3 augments the antidepressant-like effects of IMI-15 in TDS-exposed rats.

Keywords
Flinders Sensitive Line rat, major depressive disorder, phosphodiesterase type 5 inhibitor, sildenafil, time-dependent sensitisation, treatment-resistant depression.

Significant outcomes

- TDS + FSL rats present with significantly greater depressive-like behaviour compared to FSL rats.
- TDS + FSL rats display elevated hippocampal BDNF, 5-HT, NE, GSH, and GSSG levels compared to FSL rats.
- IMI-15, SIL-10, and IMI-15 + SIL-3 reversed depressive-like behaviour in TDS + FSL rats.
- Sub-chronic IMI-15, SIL-3, SIL-10 and/or IMI-15 + SIL-3 treatment reduced elevated hippocampal BDNF, 5-HT, GSH, and/or GSSG levels in TDS + FSL rats.
- SIL-3 augmented the antidepressant-like effects of IMI-15 in TDS + FSL rats.

Limitations

- This study only made use of male FSL rats.
- Hippocampal cGMP levels of the rats were not measured in this study.
- Antidepressant treatment resistance was not demonstrated with IMI-15.

Sildenafil, alleen en in kombinasie met imipramien, vertoon antidepressantagtige effekte in 'n tydafhanklike-sensitiseringgeïnduseerde (TAS-) rotmodel van behandelingsweerstandige depressie:

Agtergrond: Ten spyte van talle farmakoterapieë tot ons beskikking om major depressiewe versteuring (MDV) te behandel, heers daar uiters hoë koerse van behandelingsweerstandigheid, terwyl lastige newe-effekte algemeen voorkom. Om hierdie uitdagings die hoof te bied en behandelingsuitkomste te verbeter, is daar 'n dringende behoefte aan nuwe, verbeterde en beter verdraagde farmakologiese opsies. Bewyse dui daarop dat dit noodsaaklik is om ander gemoedsmodulerende biochemiese stelsels benewens die monoaminergiese stelsel te teiken, wat belowende alternatiewe bied. In hierdie verband verteenwoordig patofisiologiese kenmerke van MDV, met inbegrip van hiperaktiwiteit van die hipotalamus-pituïtêre-bynier-as (HPB-as), verswakte neuroplastisiteit, neuro-inflammasie, immuundisfunksie en oksidatiewe stres, teikens van belang. Die selektiewe fosfodiësterasetipe 5-inhibeerder (FDE5-inhibeerder), sildenafil, is een so 'n potensiële kandidaat, met prekliniese data wat daarop dui dat dit oor antidepressantagtige eienskappe beskik. Hoewel dit tans spekulasie is, kan hierdie aksies moontlik veranderde neuroplastisiteit behels, asook anti-inflammatoriese en antioksidant-aksies. Die terapeutiese potensiaal daarvan in behandelingsweerstandige depressie (BWD) is egter nog nie ondersoek nie.

Doel: Om te ondersoek of sildenafil, hetsy as 'n monoterapie of as aanvulling by 'n tradisionele antidepressant, antidepressantagtige biogedragseffekte kan uitoefen in 'n knaagdiermodel van BWD, en of sodanige aksies die bogenoemde prosesse behels.

Metodes: 'n Tydafhanklikesensitisering-geïnduseerde (TAS-geïnduseerde) model van BWD in Flinders Sensitive Line- (FSL-) manlike rotte is gebruik. TAS-naïewe groepe is ongestoord gelaat vanaf nageboortelike dag 40 (NGD40) tot NGD54, terwyl TAS-blootgestelde groepe vanaf nageboortelike dag NGD40 aan 'n TAS-paradigma onderwerp is. Vanaf NGD55 het rotte vir 10 dae óf draerstof (VEH), óf imipramien (15 mg/kg/dag) (IMI-15), sildenafil (3 mg/kg/dag) (SIL-3), sildenafil (10 mg/kg/dag) (SIL-10) of IMI-15 + SIL-3 ontvang. Die oopveldtoets (OVT) en geforseerde swemtoets (GST) is onderskeidelik op NGD62 en NGD63 uitgevoer. Vlakke van hippokampale breinafgeleide neurotrofiese faktor (BANF), serotonien (5-HT), norepinefrien (NE), glutatioon (GSH) en glutatioondisulfied (GSSG) is op NGD65 ontleed.

Resultate: TAS-blootstelling het immobiliteitstyd in FSL-rotte (depressiefagtige gedrag) in die GST (p = 0,0186) vergelyke met stresnaïewe FSL-rotte verleng. Subchroniese IMI-15-behandeling het immobiliteit in TAS-blootgestelde rotte verlaag, vergeleke met VEH-behandelde TAS-blootgestelde rotte (p = 0,0097), en het dus 'n behandelingsreaksie in TAS-rotte veroorsaak. SIL-10 (p = 0,0196), en IMI-15 + SIL-3 (p = 0,0005) was soortgelyk effektief, terwyl SIL-3 nie was nie. Wat belangrik is, is dat SIL-3 die antidepressantagtige effekte van IMI-15 aangevul het, met IMI-15 + SIL-3 wat groter immobiliteitverlagende vermoë getoon het, vergeleke met IMI-15, in TAS-blootgestelde rotte. Boonop het TAS-blootstelling die vlakke van hippokampale BANF (p = 0,0007), 5-HT (p < 0,0001), NE (p = 0,0227), GSH (p < 0,0001) en GSSG (p = 0,0185) in FSL-rotte verhoog, vergeleke met nie-gestresde FSL-rotte. IMI-15 (p = 0,0317) en SIL-10 (p = 0,0078) het hippokampale BANF-vlakke in TAS-blootgestelde rotte verlaag, maar het nie hippokampale 5-HT-, NE-, GSH- en GSSG-vlakke beïnvloed nie, vergeleke met VEH. Daarbenewens, vergeleke met VEH, het subchroniese SIL-3-behandeling hippokampale GSH-vlakke (p = 0,0018) van TAS-blootgestelde rotte verlaag sonder om hippokampale BANF-, 5-HT-, NE- en GSSG-vlakke te verander. Subchroniese IMI-15 + SIL-3-behandeling het vlakke van hippokampale BANF (p = 0,0024), 5-HT (p = 0,0115), GSH (p < 0,0001) en GSSG (p = 0,0002) by TAS-blootgestelde rotte vergeleke met VEH, maar sonder om hippokampale NE-vlakke te beïnvloed. SIL-3 het die 5-HT- (p = 0,0115) en GSSG-verlagende (p = 0,0002) effekte van IMI-15 versterk, met IMI-15 + SIL-3 wat statistiese betekenisvolheid in TAS-blootgestelde rotte bereik het, maar wat nie met IMI-15 nie of SIL-3 alleen die geval was nie. Laastens het SIL-3 die GSH-verlagende werking van IMI-15 (p = < 0,0001) versterk, vergeleke met SIL-3 alleen (p = 0,0018), terwyl IMI-15 op sigself geen sodanige effekte in TAS-blootgestelde rotte gehad het nie.

Gevolgtrekking: TAS + FSL rotte het depressiewe gedrag en bewyse van oksidatiewe stres getoon, maar paradoksaal het hulle verhoogde hippokampale monoamiene en BANF gehad. Hierdie oënskynlik teenregulerende werking van BANF word herbevestig deur IMI-15, SIL-10 en IMI-15 + SIL-3, wat verhoogde BANF en depressiefagtige gedrag in TAS + FSL-rotte omgekeer het. Ook het SIL-3 slegs sommige redoksveranderinge omgekeer, terwyl IMI-15 + SIL-3 serotonergiese en alle redoksveranderinge omgekeer het. SIL-3 het die antidepressantagtige effekte van IMI-15 in TAS-blootgestelde rotte aangevul.

Sleutelwoorde
Behandelingsweerstandige depressie, Flinders Sensitiewe Lyn-rot, fosfodiësterase tipe 5-inhibeerder, major depressiewe versteuring, sildenafil, Sprague-Dawley-rot, tydafhanklike sensitisering.

Betekenisvolle uitkomste

- TAS + FSL-rotte presenteer met 'n beduidend groter mate van depressiefagtige gedrag vergeleke met FSL-rotte.
- TAS + FSL-rotte vertoon verhoogde hippokampale BANF-, 5-HT-, NE-, GSH- en GSSG-vlakke vergeleke met FSL-rotte.
- IMI-15, SIL-10 en IMI-15 + SIL-3 het depressiefagtige gedrag in TAS + FSL-rotte omgekeer.
- Subchroniese behandeling met IMI-15, SIL-3, SIL-10 en/of IMI-15 + SIL-3 het verhoogde hippokampale BANF-, 5-HT-, GSH- en/of GSSG-vlakke in TAS + FSL-rotte verlaag.
- SIL-3 het die antidepressantagtige effekte van IMI-15 in TAS + FSL rotte aangevul.

Beperkings

- Hierdie studie het slegs van manlike FSL-rotte gebruik gemaak.
- Die hippokampale sGMF-vlakke van die rotte is nie in hierdie studie gemeet nie.
- Weerstandigheid teen antidepressantbehandeling is nie met IMI-15 gedemonstreer nie.

Introduction

Major depressive disorder (MDD) is a serious, debilitating and highly disabling mood disorder (Bylund & Reed, 2007; Reierson et al., 2011; National Institute of Mental Health, 2023) with a characteristically chronic, relapsing or recurrent course of illness (Preboth, 2000; Hardeveld et al., 2010; Reierson et al., 2011; Ten Have et al., 2018; Verhoeven et al., 2018). In addition to being the leading cause of disability world-wide (Preboth, 2000; Friedrich, 2017), MDD is a large contributor to the global burden of disease (Vos et al., 2017; World Health Organization, 2023b). MDD is indiscriminate of age, sex, race, and socio-economic status (Hasin et al., 2005; Forman-Hoffman & Viswanathan, 2018; Hasin et al., 2018; Ghandour et al., 2019). According to estimates, as many as 322 million individuals worldwide, or 4,4% of the global population, suffer from MDD (World Health Organization, 2017), making it the most common psychiatric disorder (Willner et al., 2013; Gupta et al., 2018).

Treatment-resistant depression (TRD) is an extremely severe, difficult-to-manage and persistent form of MDD, having a high global prevalence (Fagiolini & Kupfer, 2003; Rush et al., 2008; Al-Harbi, 2012; Cleveland Clinic, 2023b). Although known to develop in susceptible individuals exposed to traumatic events (Brand & Harvey, 2017), TRD has been associated with a number of co-morbidities, including anxiety disorders, post-traumatic stress disorder (PTSD), and psychosis (Kornstein & Schneider, 2001; Hauksson et al., 2017; Albott et al., 2018; Lucchese et al., 2021). Symptoms of TRD are frequently akin to those of MDD, yet individuals are more inclined to experience greater symptom severity, major depressive episodes (MDEs) that last longer and a larger number of MDEs during their lifetimes (Cleveland Clinic, 2023a). Individuals with TRD not only experience an unabating depressed mood (feeling sad, irritable and/or empty) and anhedonia (loss of pleasure and/or interest in daily activities), but also several other less distinctive albeit burdensome symptoms most of the day and nearly every day for at least two weeks, such as changes in appetite and sleeping patterns (Fava & Kendler, 2000; American Psychiatric Association, 2013; National Institute of Mental Health, 2023; World Health Organization, 2023d).

Expectedly, those affected by MDD and TRD usually encounter significant functional impairment, most notably impaired social, cognitive and/or occupational functioning (Preboth, 2000; Harley et al., 2008; Al-Harbi, 2012; Lam et al., 2013; Gregory et al., 2020; Li, 2023; World Health Organization, 2023a). These add to the substantial financial burden on the affected individuals, their families, and society at large (Lam et al., 2013; Greenberg et al., 2015; Williams et al., 2017; McCrone et al., 2018; Li, 2023). Moreover, a markedly reduced quality of life is seen in individuals suffering from mood disorders, including MDD and TRD (Papakostas et al., 2004; IsHak et al., 2011; Lépine & Briley, 2011; Williams et al., 2017; Lex et al., 2019), and they have an increased risk of suicide compared to their non-depressed counterparts (Hawton et al., 2013; Bergfeld et al., 2018). In fact, globally more than 700 000 suicide-related deaths are reported annually (World Health Organization, 2023c).

Apart from non-pharmacological therapeutic options for TRD, such as cognitive behavioural therapy (CBT) and electroconvulsive therapy (ECT), pharmacological strategies are aimed at optimising, combining, and switching pharmacological classes of traditional antidepressants, administering novel psychoactive agents like ketamine and psilocybin, and augmenting existing pharmacotherapy. The latter generally comprises the addition of a second medication, not conventionally considered an antidepressant, such as the mood-stabiliser lithium, or a second-generation antipsychotic like olanzapine (Voineskos et al., 2020). Remarkably, present-day antidepressants do not satisfy all the clinical requirements for managing MDD,

let alone TRD, e.g., troublesome side effects, delayed onset of action and poor response. In fact, one-third of MDD patients do not achieve remission after receiving a series of conventual antidepressant trials even at optimal dose and duration of treatment (Rush et al., 2006; Rosenzweig-Lipson et al., 2007; Al-Harbi, 2012; Zorumski et al., 2015). The latter are regarded as being treatment resistant. Consequently, studies into novel, better-tolerated pharmacotherapies that are more effective in resistant types of MDD are warranted.

Conventional antidepressants predominantly modulate central monoaminergic signalling (Malhi et al., 2013; Willner et al., 2013; Boku et al., 2018; Duarte-Silva et al., 2020), specifically elevating the synaptic levels of serotonin (5-HT), norepinephrine (NE) and/or dopamine (DA) (Goldberg et al., 2014). This approach to treatment describes the underpinnings of the monoaminergic hypothesis of MDD (Schildkraut, 1965; Hirschfeld, 2000). Nevertheless, the high prevalence of resistance to these agents (Duarte-Silva et al., 2020) emphasizes that the pathophysiology underlying MDD is far more complex and diverse than expected, requiring different and novel mechanistic strategies.

In this regard, impaired neuroplasticity, including structural plasticity and functional synaptic plasticity, plays a central role in the neuropathology of MDD, as well as in the action of antidepressants (Duman, 2002; Duman & Monteggia, 2006; Duman et al., 2008). These processes in turn are regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Carlson et al., 2006; Kunugi et al., 2010). HPA axis hyperactivity, and by implication hypersecretion of circulating adrenocorticotropic hormone (ACTH) and glucocorticoids, is frequently observed in MDD (Plotsky et al., 1998; Varghese & Brown, 2001; Barden, 2004; Pariante & Lightman, 2008). Importantly, successful pharmacotherapy is associated with a reversal of the aforementioned HPA axis changes (Plotsky et al., 1998; Varghese & Brown, 2001; Barden, 2004; Pariante & Lightman, 2008) as well as reversal of hippocampal atrophy (Warner-Schmidt & Duman, 2006). TRD is associated with abrogated HPA axis negative feedback (Hornig-Rohan et al., 1996; Markopoulou et al., 2009; Markopoulou, 2013; Caraci et al., 2018), i.e., hypercortisolaemia, while its lack of response to traditional agents suggests recalcitrant neuroplastic mechanisms that are non-responsive to such treatment (Harvey et al., 2003).

An earlier hypothesis put forward the role of the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) system in treatment non-response in MDD (Harvey, 1996; Harvey et al., 2003). Importantly, the selective phosphodiesterase type 5 (PDE5) inhibitors, such as sildenafil and tadalafil, which enhance cGMP-protein kinase G (PKG) signalling, have antidepressant-like properties in translational rat models of MDD (Brink et al., 2008; Liebenberg et al., 2010a; Tomaz et al., 2014; Wang et al., 2014; Socała et al., 2016). It has been demonstrated that at higher doses of 10 or 20 mg/kg sildenafil, but not a lower dose of 3 mg/kg, addition of a centrally active anticholinergic drug is required to unmask the antidepressant activity of the PDE5 inhibitor (Brink et al., 2008; Liebenberg et al., 2010a). Of note, the tricyclic antidepressant imipramine is well known to possess potent inherent anticholinergic activity, suggesting a plausible augmentation strategy when combined with PDE5 inhibitors. The cGMP/PKG signalling pathway plays a pivotal role in regulating the downstream cyclic adenosine monophosphate (cAMP)-response element binding protein/brain-derived neurotrophic factor (CREB/BDNF) signalling cascade. Hence, amplification of cGMP/PKG signalling by PDE5 inhibitors would increase CREB/BDNF signalling and ultimately neuroplasticity (Feil et al., 2005; Dhir & Kulkarni, 2007; Brunoni et al., 2008; Kapczinski et al., 2008; Puzzo et al., 2008; Reierson et al., 2011; Wang et al., 2014).

In addition, PDE5 plays an intimate role in regulating cellular redox status, which in itself will have indirect effects on synaptic plasticity, oxidative stress and correct functioning of the nervous system (Reay, 2010; Savas et al., 2010; Verit et al., 2010; Thakur et al., 2013; Tomaz et al., 2014; Sikandaner et al., 2017). Furthermore, MDD is associated with a biological imbalance between the levels of reactive oxygen species (ROS) and those of antioxidants, e.g., glutathione (GSH), leading to oxidative stress (Bakunina et al., 2015) and altered biomolecules such as monoamines (Brand et al., 2015; Mokoena et al., 2015). The failure of cells to adapt to altered redox homeostasis and the subsequent occurrence of cell death are key factors implicated in the aetiology of MDD (Bakunina et al., 2015).

Finally, a recent study in our laboratories (Saayman et al., 2024) demonstrated in an adrenocorticotropic hormone- (ACTH-) induced rodent model of TRD that an imipramine plus sildenafil combination offered a more effective augmentation strategy to reverse TRD than a escitalopram plus sildenafil combination. Since escitalopram is devoid of anticholinergic properties, this tentatively supports the above notion, although further investigation with imipramine and sildenafil in a different animal model of TRD are required to verify this claim. In addition, although several tricyclic antidepressants are known to possess antimuscarinic activity (see the significance thereof explained above), including a recent study in an ACTH-induced model of TRD (Saayman et al., 2024), imipramine is well studied in our laboratory and hence an appropriate antidepressant to use.

Validated animal models that have translational value to humans are routinely used by pre-clinical researchers to identify drugs with the potential to treat mood disorders, such as TRD (Pereira et al., 2019). Using a gene-x-environmental model of TRD, based on the exposure of Flinders Sensitive Line (FSL) rats to a time-dependent sensitisation (TDS) model of stress/re-stress (Brand & Harvey, 2017), we studied the dose-dependent antidepressant-like effects of sildenafil alone and in combination with a known monoaminergic antidepressant following sub-chronic treatment. In addition, the associated depressive-like behavioural changes were correlated to hippocampal BDNF, monoamines, glutathione (GSH), and glutathione disulphide (GSSG) levels. We hypothesised that TDS-exposed FSL rats would show non-response to imipramine, but partial response to sub-chronic low-dose sildenafil monotherapy and full response to sub-chronic high-dose sildenafil monotherapy and imipramine plus low-dose sildenafil combination therapy versus

treatment naïve TDS-exposed FSL rats. Finally, we predicted that imipramine plus low-dose sildenafil will demonstrate augmentation versus either drug alone.

Materials and methods

Animals

Male FSL rats (n = 60) were bred, supplied, and housed at the Animal Centre (Vivarium) of the Pre-Clinical Drug Development Platform (PCDDP) of the South African Department of Science and Innovation (DSI) and North-West University (NWU), South Africa. The Animal Centre is registered with the South African Veterinary Council (SAVC) (reg. no.: FR15/13458) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) (international file #1717). The original FSL rat colonies were acquired from Dr. David H. Overstreet, University of North Carolina, Chapel Hill, North Carolina, United States of America. All the rats used in this study were group-housed (2 rats/cage) in individually ventilated polysulphone cages (395 x 346 x 213 mm (l x w x h)) under conditions of constant room temperature (22 ± 1°C), humidity (55 ± 10%) and positive air pressure. The Animal Centre air was exchanged with fresh uncirculated air 16 to 18 times per hour, and high-efficiency particulate air (HEPA) filters controlled the air quality. A 12-hour light/dark cycle was always maintained (lights were switched on between 06:00 and 18:00). Cages were cleaned, and bedding (composed of chipped corncob) was replaced weekly. All the rats had constant access to a polyvinyl chloride (PVC) tunnel shelter and nesting material (consisting of paper towels) in their home cages as environmental enrichment. Food (standard rat chow) and tap water were supplied *ad libitum*. Rats and experimental facilities were protected from outside noise.

Drug treatments

All the rats were weighed daily during the sub-chronic treatment period (over 10 days) of this study, and the correct amount of the individual drugs was calculated accordingly for every dose administered to each rat. To ensure product stability, the individual drug powders were kept in their original containers and according to the specifications of the manufacturer. All the drug powders were dissolved in phosphate-buffered saline (PBS) and then administered to the rats via subcutaneous (SC) injection. Drug powders were dissolved in a maximum of 0,25 ml of PBS for each dose, ensuring that the powders completely dissolved upon visual inspection, using the smallest possible volume to limit any discomfort of injecting larger volumes via the SC route. Control rats were injected with vehicle control (VEH), which consisted of PBS alone, in the same manner as drug-treated rats.

The following drugs were administered between 08:00 and 10:00 as monotherapies and some also as combination therapies: (1) imipramine hydrochloride (Sigma-Aldrich®, Schnelldorf, Germany) at 15 mg/kg/day (IMI-15) (Sales et al., 2011; Pereira et al., 2019) and (2) sildenafil citrate (Sigma-Aldrich®, Schnelldorf, Germany) at 3 mg/kg/day (SIL-3) or 10 mg/kg/day (SIL-10) (Liebenberg et al., 2010a). The dosages chosen for sildenafil and imipramine were verified in an aforegoing sister paper (Saayman et al., 2024). Briefly, that paper identified high-dosage sildenafil (10 mg/kg), low-dosage sildenafil (3 mg/kg) and imipramine (15 mg/kg) for use in this study.

Experimental design

Figure 1 is a schematic illustration of the study layout. On PND21, all the rat pups were weaned and randomly allocated to home cages. All the rats were individually marked on PND21 by clipping their ears. Following their allocation to home cages, the rat pups were randomly divided into a TDS-naïve and TDS-exposed group comprising 12 and 48 rats, respectively. Rat pups were subsequently randomly divided into various sub-chronic treatment groups comprising 12 rats each.

Starting on PND40, TDS-exposed rats were subjected to the TDS paradigm (see section 2.3.1.) until PND54, whereas TDS-naïve rats were left undisturbed. The various sub-chronic treatments followed between PND55 and PND64. On the evening of PND61, all rats were acclimatised to an open field test (OFT) arena for 10 min under red light (80 lux). The OFT test procedure was performed 24 hours later, on the evening of PND62, followed by the forced swim test (FST) on the evening of PND63. The OFT test procedure was conducted in order to control for any effects of treatment on general locomotor activity that may confound interpretation of the FST (Saayman et al., 2021). All the rats were then humanely euthanised on PND65 (at least 36 hours following the FST to eliminate any short-term effects of swim stress on biochemical marker levels), whereafter brain (hippocampus) samples were collected and stored at –80 ˚C for subsequent biochemical analyses. Finally, levels of neurochemical markers, namely BDNF, 5-HT, NE, GSH and glutathione disulphide (GSSG), were measured in the hippocampi.

Rodent model of treatment-resistant depression

A previously established gene-x-environment rat model of TRD was used. Here TRD-like behaviour is induced in FSL rats following exposure to a post-traumatic stress disorder (PTSD) paradigm (see section 2.4.1.) (Brand, 2017; Brand & Harvey, 2017).

Time-dependent sensitisation (TDS) paradigm

TDS is an animal model of PTSD (Harvey et al., 2006). In this regard, rats present with notable bio-behavioural alterations after experiencing a severely traumatic event that perpetuates over time following exposure to subsequent contextual reminders. These bio-behavioural alterations display a time-dependent sustaining or worsening in the absence of the initiating stressor (Yehuda & Antelman, 1993; Harvey et al., 2006). Figure 2 illustrates the TDS paradigm implemented in this study.

The TDS paradigm comprised an initial severe traumatic experience, followed one and two weeks later by contextual reminders of the initial trauma (see Figure 2). In short, a

translational genetic rodent model of MDD, namely the stress sensitive FSL rat, was subjected to an acute single prolonged stress (SPS) sequence on postnatal day 40 (PND40), which consisted of three types of stressors, namely a somatosensory (restraint), psychological (forced swimming with brief underwater submersion) and complex (inescapable exposure to ether vapours until loss of consciousness occurred) stressor. Then, the rats were exposed to a single contextual reminder (CR) on PND47 and PND54, which involved restraint stress as noted above (Harvey et al., 2006). The somatosensory, psychological and complex stressors are discussed in sections 2.4.1.1., 2.4.1.2. and 2.4.1.3, respectively.

Somatosensory stressor (restraint)

The rats were individually placed into Perspex® compartments for 2 hours on PND40, with the tailgates adjusted for each rat to ensure that the rats were well restrained without impairing perfusion to their limbs. The same procedure was repeated on PND47 and PND54 (Harvey et al., 2006; Brand & Harvey, 2017). The researcher was inside the experimental room with the restrained rats for the duration of the 2 hours to be able to monitor and intervene should it have been necessary, viz., in case rats experienced distress exceeding that which was anticipated.

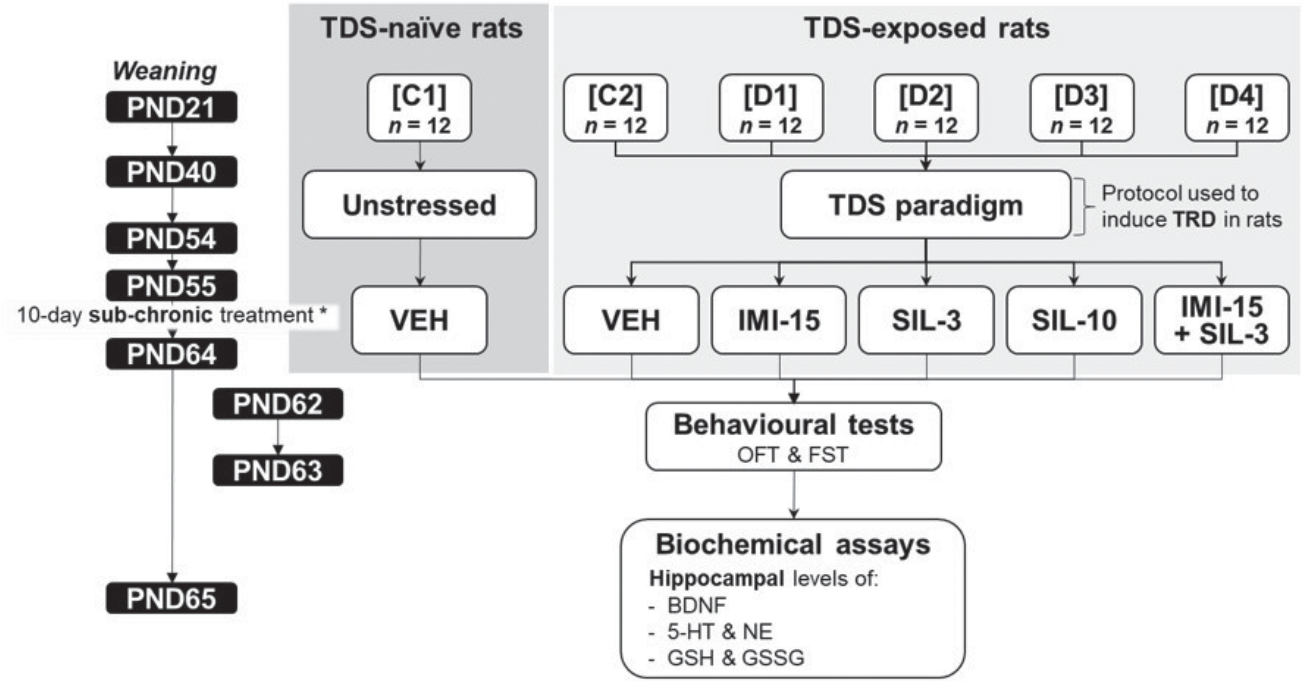

Figure 1: A schematic illustration of the study layout. BDNF: brain-derived neurotrophic factor. FST: forced swim test. GSH: glutathione. GSSG: glutathione disulphide. 5-HT: serotonin. IMI-15: imipramine hydrochloride (15 mg/kg/day). n: number of rats per treatment group. NE: norepinephrine. OFT: open field test. PND: postnatal day. SIL-3: sildenafil citrate (3 mg/kg/day). SIL-10: sildenafil citrate (10 mg/kg/day). TDS: time-dependent sensitisation. TRD: treatment-resistant depression. VEH: vehicle control. *Only 1 VEH or drug dose was administered daily during this period (with the final dose administered 24 hours before the biochemical analyses were performed).

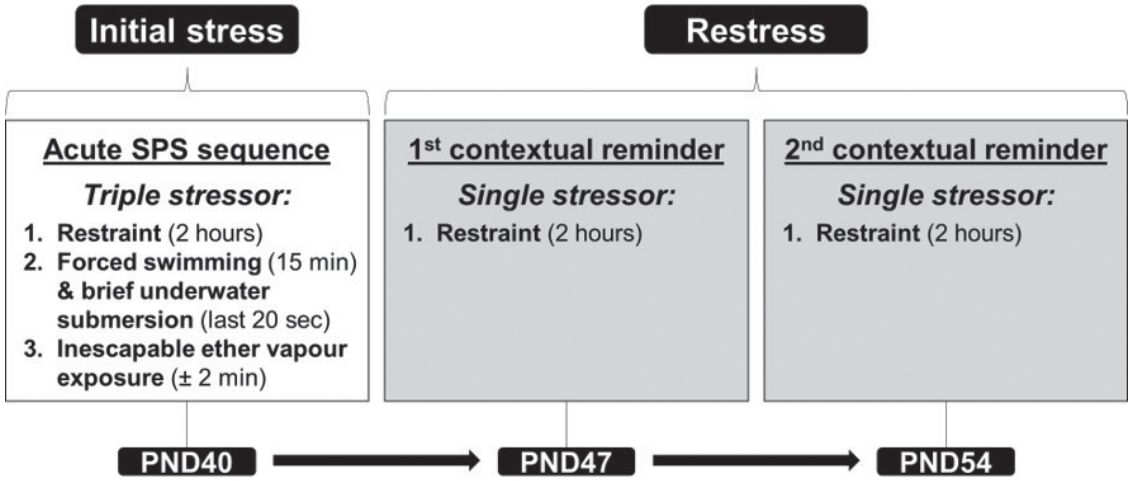

Figure 2: A schematic illustration of the TDS paradigm used to induce TRD in FSL rats. PND: postnatal day. SPS: single prolonged stress. Adapted from (Harvey et al., 2006).

Psychological stressor (forced swimming and brief underwater submersion)

Immediately after being subjected to the somatosensory stressor on PND40 (see section 2.4.1.1.), the rats were individually placed into inescapable Perspex® cylinders with dimensions 20 x 100 cm (*d* x *h*), filled with 40 cm of ambient water (25 ± 1°C), and allowed to swim for 15 min. For the final 20 seconds, the rats were forcefully submerged underwater. The rats were subsequently removed from the Perspex® cylinders, gently dried with towels and returned to their home cages for 15 min to recover (Harvey et al., 2006; Brand & Harvey, 2017). Importantly, any potential conditioned response to forced swim stress during behavioural testing in the adapted version of the forced swim test (FST) on PND63 was unlikely, as forced swimming that formed part of the TDS paradigm (implemented on PND40) was conducted 23 days prior to behavioural testing in the FST (Brand & Harvey, 2017). The investigator was inside the experimental room with the swimming rats for the duration of the 15 min, which enabled him to monitor and intervene should it have been necessary, viz., in case rats stopped attempting to keep their heads above water and were at risk of drowning.

Complex stressor (inescapable exposure to ether vapours)

Once the 15 min recovery period elapsed after the psychological stressor (see section 2.4.1.2.), still on PND40, rats were subjected to the vapours of ether until loss of consciousness (for a duration of approximately 2 min). Inside a custom-designed fume hood, 5 ml of 100% diethyl ether was poured onto a paper towel, which was then placed at the bottom of a 5 L sealed plastic container. Subsequently, rats were individually placed on a raised metal platform within the plastic container to avoid direct contact with the ether. Immediately upon losing consciousness, the rats were removed from the plastic container and returned to their home cages, where they were observed until full consciousness was regained (Harvey et al., 2006; Brand & Harvey, 2017).

Contextual reminders

After completion of the acute SPS sequence on PND40 described above (see sections 2.4.1.1. to 2.4.1.3.), all the rats were returned to their group-housed home cages in the holding room and left undisturbed until PND47, when the first re-exposure to the somatosensory stressor (contextual reminder) took place. Immediately thereafter, the rats were again returned to their home cages in the holding room and left undisturbed. The second and final re-exposure to the contextual reminder was subsequently carried out on PND54 (Harvey et al., 2006; Brand & Harvey, 2017).

Behavioural tests

A study previously conducted in our laboratories indicated that the aforegoing behavioural tests do not impact the results of subsequent consecutive tests when the least stressful behavioural test is performed first, followed by the more stressful tests in order of increasing stressfulness (Mokoena et al., 2015). As a result, the OFT was conducted 24 hours before the FST. Behavioural tests were performed during the dark cycle (between 18:00 and 06:00), considering that rats are nocturnal animals. In fact, behavioural tests only started 1 hour following the start of the dark cycle (at 19:00) to allow for the initial foraging and activity of rats. The equipment and methods used in this study to conduct the behavioural tests (the OFT and FST) are described below (see sections 2.5.1. and 2.5.2.).

Open field test (OFT)

Based on the observation that rodents are naturally inquisitive animals and inclined to explore novel environments, the OFT test procedure is used to measure their general locomotor activity. General locomotor activity can be defined as a measure of rodents' ability to move around and negotiate their environment (Schoeman et al., 2017). Importantly, results obtained from the OFT test procedure concerning general locomotor activity may be influenced by rodents having an elevated anxiety-like behaviour, which is caused by placing them in a novel environment (Misslin & Cigrang, 1986), considering that more anxious rodents tend to remain motionless in a corner or beside a wall of the OFT arena (where they perceive to be protected by the walls of the OFT arena and consequently feel safe) for longer durations relative to those that are less anxious (Ramos et al., 1997; Prut & Belzung, 2003; Hiroi & Neumaier, 2006). Therefore, to reduce the anxiety experienced by rats resulting from being placed in novel surroundings during the OFT test procedure, they were acclimatised to an OFT arena 24 hours before the OFT test procedure was performed.

Four 100 x 100 x 45 cm (*l* x *w* x *h*) square arenas with opaque black walls and a separate video camera situated directly above each OFT arena comprised the OFT apparatus. The OFT test procedure was carried out under red light (80 lux) on PND62 and as previously described for our laboratories (Schoeman et al., 2017; Saayman et al., 2021). Briefly, rats were individually placed in the centres of the OFT arenas and allowed to explore their new surroundings for 5 min while being recorded by video cameras situated directly above the OFT arenas. The rats were returned to their home cages after each test session, and the OFT arenas were then wiped clean with a 10% ethanol solution to remove any olfactory cues during subsequent test sessions. Using EthoVision XT 14 software (Noldus Information Technology BV, Wageningen, Netherlands), the video recordings were later scored, and the total distance moved (cm) during the OFT test procedure was used as a measure of the general locomotor activity of the rats.

Forced swim test (FST)

The FST is based on the observation that when rodents are placed into inescapable, water-filled cylinders, they will initially attempt to escape through escape-directed behaviour (Armario et al., 1988), whereafter they develop an immobile posture (with depressive-like rodents developing an immobile posture faster compared to healthy controls). Immobility in the FST has therefore been associated with failing to persevere in escape-

directed behaviour (behavioural despair), reflecting depressive-like behaviour (Porsolt et al., 1977; Porsolt, 1979; Lucki, 1997; Petit-Demouliere et al., 2005). Consequently, the FST is commonly performed in rodents to evaluate antidepressant-like activity over a broad spectrum of antidepressants (Borsini & Meli, 1988). Crucially, FSL rats inherently present with elevated depressive-like behaviour (an elevated time spent immobile) in the FST, and a pre-conditioning swim session 24 hours before the FST is unwarranted for these rats to display behavioural despair during the FST (Overstreet et al., 2005; Saayman et al., 2021).

The FST apparatus comprised four 20 x 100 cm (*d* x *h*) cylindrical tanks positioned adjacent to one another, each filled to a depth of 30 cm with ambient water (maintained at 25 ± 1 ˚C), and a video camera situated directly in front of the four FST cylinders. The FST was carried out under red light (80 lux) on PND63 and as previously described for our laboratories (Schoeman et al., 2017; Saayman et al., 2021). Briefly, rats were individually placed into the water-filled FST cylinders and permitted 7 min for swimming while being video recorded. The rats were removed from the water-filled FST cylinders immediately after the 7 min elapsed, gently dried with a towel and returned to their home cages. After every FST trial, the water in the FST cylinders was replaced with clean water, eliminating any effects that alarm substances may have had on rats during subsequent FST trials (Abel & Bilitzke, 1990).

The researcher had a clear view of all the rats for the entire duration of the FST, enabling intervention should it have been necessary, viz., in case rats could not keep their heads above water and were at risk of drowning. Using EthoVision XT 14 software (Noldus Information Technology BV, Wageningen, Netherlands), the video recordings were scored, and the total time spent immobile (sec) during the FST was used as a measure of depressive-like behaviour of rats. However, the first and last minutes of the FST were not scored (so that a total of 5 min were scored), thereby maximising the accuracy of the results.

Biochemical assays

Brain tissue collection and preparation

After sacrifice, whole brains of rats were immediately extracted and placed in ice-cold PBS. Hippocampi were promptly dissected out on an ice-cooled dissection slab before being snap frozen at –80˚C until assays of hippocampal BDNF, 5-HT, NE, GSH and GSSG were performed (Harvey et al., 2006; Brand & Harvey, 2017; Steyn et al., 2018; Saayman et al., 2021).

Hippocampal brain-derived neurotrophic factor (BDNF) assay

BDNF was assayed using enzyme-linked immunosorbent assay (ELISA) kits (catalogue no.: E-EL-R1235; Elabscience Biotechnology Inc.), according to the instructions of the manufacturer. Concentrations of BDNF were extrapolated from a standard curve and expressed as pg/ml wet tissue weight (Steyn et al., 2018; Saayman et al., 2021).

Hippocampal serotonin (5-HT), norepinephrine (NE), glutathione (GSH), and glutathione disulphide (GSSG) assay

According to previous methods, hippocampal tissue samples were assayed for 5-HT, NE, GSH and GSSG using liquid chromatography-mass spectrometry (LC-MS) (Moriarty et al., 2011; Squellerio et al., 2012; Fuertig et al., 2016; Wojnicz et al., 2016; Wang et al., 2019).

Statistical analyses

Power analyses were conducted, establishing the minimum number of rats required per treatment group included to ensure statistically meaningful results, using G*power version 3.1.9.2. In instances where a one-way analysis of variance (ANOVA) was conducted, power calculations were performed considering the omnibus test (*F* statistic). The standardised effect size was defined as medium ($F = 0{,}25$) to large ($F = 0{,}40$). In all instances, power calculations were performed considering a type-I error rate of 5% ($\alpha = 0{,}05$), and the threshold for type-II error considered was 20% ($1\text{-}\beta \geq 0{,}8$).

GraphPad Prism® version 8 for Windows (GraphPad Prism® software, Version 8.0, San Diego California, United States of America) was used to perform all the statistical analyses and to create graphical representations. To screen for outliers, Grubbs' test was conducted on all data sets (with $\alpha = 0{,}05$ accepted as significant). The Shapiro-Wilk's and Levene's tests were performed to test for normality of distribution and homogeneity of variances, respectively (with $p < 0{,}05$ accepted as a violation of both assumptions). An unpaired Student's t-test (data distributed normally) or Mann-Whitney U-test (data not distributed normally) was utilised to establish the effects of TDS paradigm exposure on the bio-behaviour parameters compared to the TDS-naïve control group. An Ordinary one-way ANOVA (multiple comparisons for data distributed normally) or Kruskal-Wallis one-way ANOVA (multiple comparisons for data not distributed normally) was conducted to establish the effects of different sub-chronic treatments in TDS-exposed rats. To compare the various sub-chronic drug treatment groups to the VEH treatment group in TDS-exposed rats, Ordinary one-way ANOVAs were followed by a Dunnett's post-hoc test for multiple comparisons, while Kruskal-Wallis one-way ANOVAs were followed by a Dunn's test. Data are presented as the mean ± standard error of the mean (SEM), and statistical significance was set at $p \leq 0{,}05$ for all comparisons.

To determine practical (clinical) significance of effect magnitude, the unbiased Cohen's *d* value (*d*) was utilised to calculate effect magnitude of interactions and intergroup differences (with a 95% confidence interval (CI) of the effect magnitude). Only medium and large effect sizes ($d \geq 0{,}6$ and $d \geq 0{,}8$, respectively) were considered significant. These were calculated using Exploratory Software for CIs (Cohen, 1988).

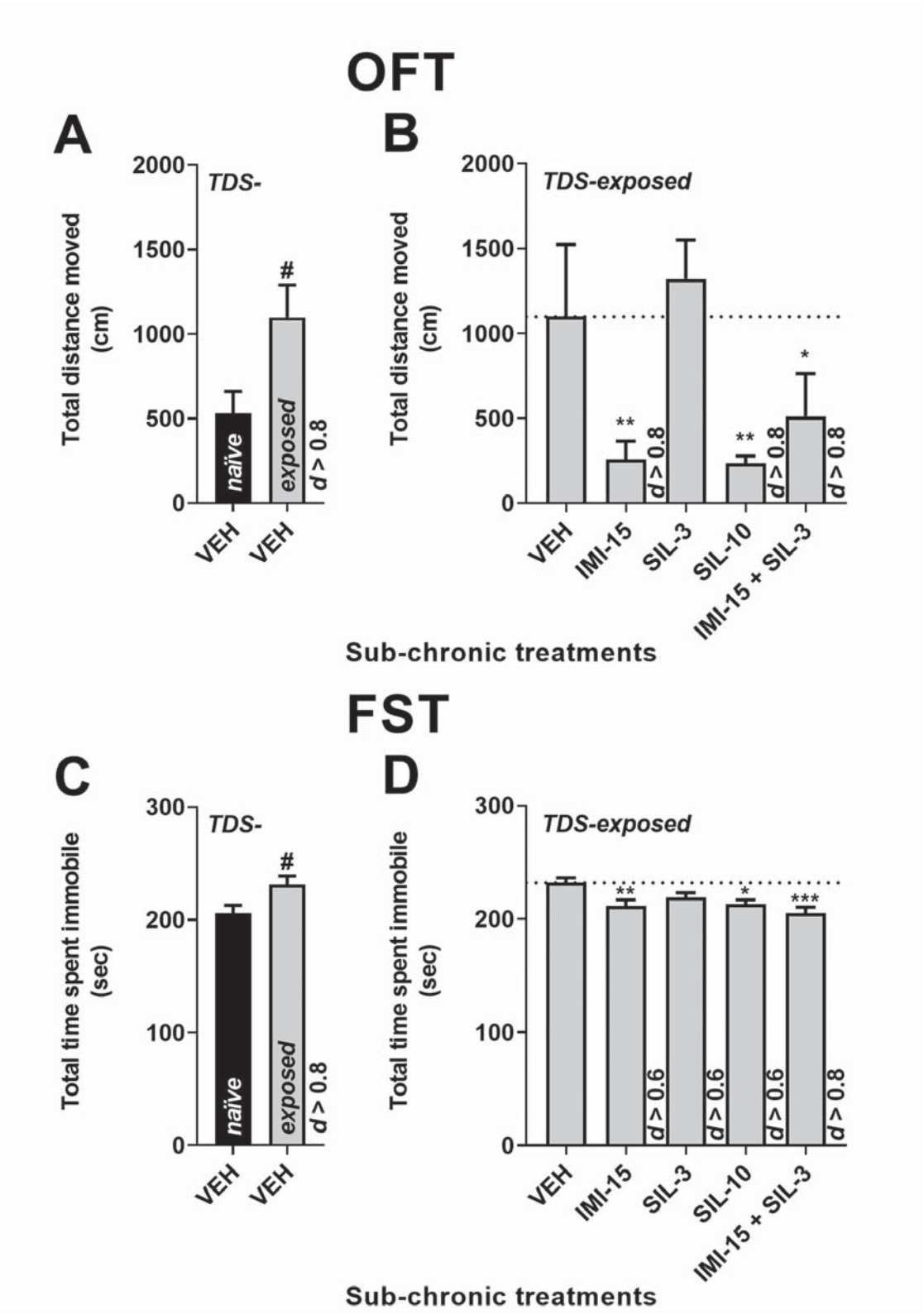

Figure 3: A graphical representation of the OFT and FST data. A: Distance moved by FSL rats after being left unstressed or exposed to the TDS paradigm and subsequently receiving sub-chronic VEH treatment. **B**: Distance moved by FSL rats after being exposed to the TDS paradigm and subsequently receiving sub-chronic VEH or drug treatment, as indicated. **C**: Time spent immobile by FSL rats after being left unstressed or exposed to the TDS paradigm and subsequently receiving sub-chronic VEH treatment. **D**: Time spent immobile by FSL rats after being exposed to the TDS paradigm and subsequently receiving sub-chronic VEH or drug treatment, as indicated. All group sizes are equal (n = 12). Data points represent the mean ± SEM. Statistical analyses are reported in the text with # $p < 0,05$ and d vs the TDS-naïve + VEH treatment group in **A**, * $p < 0,05$, ** $p < 0,01$ and d vs the TDS-exposed + VEH treatment group in **B**, # $p < 0,05$ and d vs the TDS-naïve + VEH treatment group in **C**, and * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ and d vs the TDS-exposed + VEH treatment group in **D**. d: unbiased Cohen's d-value. FST: forced swim test. IMI-15: imipramine hydrochloride (15 mg/kg/day). OFT: open field test. SIL-3: sildenafil citrate (3 mg/kg/day). SIL-10: sildenafil citrate (10 mg/kg/day). TDS: time-dependent sensitisation. VEH: vehicle control.

Results

Behavioural analysis

Data obtained from the behavioural tests performed on PND62 and PND63, once the rats had been left unstressed or exposed to the TDS paradigm and all the VEH and drug treatments had been administered, are presented below (see section 3.1.1.).

Distance moved and time spent immobile

Figure 3 depicts the total distance moved in the OFT and time spent immobile in the FST by FSL rats after they were left unstressed or exposed to the TDS paradigm and subsequently received sub-chronic (over 10 days) VEH or drug treatment. FST data are corrected for treatment-induced alterations in the general locomotor activity of rats using analyses of covariance (ANCOVAs).

An unpaired Student's t-test revealed that TDS exposure significantly elevates the distance moved by FSL rats in the OFT compared to non-stressed FSL rats (p = 0,0231, d = 0,96) (see Figure 3A). An Ordinary one-way ANOVA test for multiple comparisons in TDS-exposed FSL rats indicates statistically significant differences between the various sub-chronic treatment groups regarding distance moved in the OFT ($F_{(4, 52)}$ = 10,64, p < 0,0001). Dunnett's post-hoc test for multiple comparisons showed, in TDS-exposed FSL rats, that sub-chronic IMI-15 (p = 0,0010, d = 1,63), SIL-10 (p = 0,0010, d = 1,67) and IMI-15 + SIL-3 (p = 0,0256, d = 1,03) treatments significantly reduces the distance moved in the OFT compared to VEH (see Figure 3B). An unpaired Student's t-test revealed that TDS exposure in FSL rats significantly elevates the time spent immobile in the FST compared to non-stressed FSL rats (p = 0,0186, d = 0,97) (see Figure 3C). An Ordinary one-way ANOVA test for multiple comparisons in TDS-exposed FSL rats indicates statistically significant differences between the various sub-chronic treatment groups regarding time spent immobile in the FST ($F_{(4, 55)}$ = 4,802, p = 0,0021). Dunnett's post-hoc test for multiple comparisons showed, in TDS-exposed FSL rats, that sub-chronic IMI-15 (p = 0,0097, d = 0,64), SIL-10 (p = 0,0196, d = 0,61) and IMI-15 + SIL-3 (p = 0,0005, d = 1,02) treatments significantly reduces the time spent immobile in the FST compared to VEH-treated TDS-exposed FSL rats (see Figure 3D).

Rat biochemical marker levels

Data obtained from the various biochemical assays conducted on PND65, once the rats had been left unstressed or exposed to the TDS paradigm, all the VEH and drug treatments had been administered and behavioural tests had been performed, are presented below (see section 3.2.1. to 3.2.3.).

Hippocampal brain-derived neurotrophic factor (BDNF) levels

Figure 4 depicts the total hippocampal BDNF levels of FSL rats after they were left unstressed or exposed to the TDS paradigm and subsequently received sub-chronic VEH or drug treatment.

An unpaired Student's t-test revealed that TDS exposure significantly elevates the hippocampal BDNF levels of FSL rats compared to non-stressed FSL rats (p = 0,0007, d = 1,55) (see

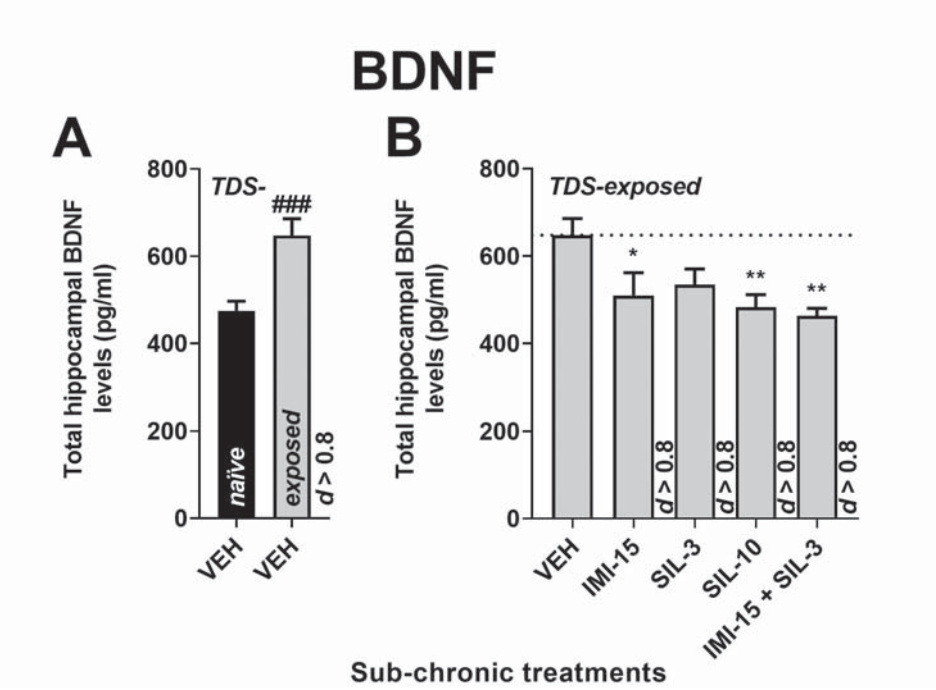

Figure 4: A graphical representation of the hippocampal BDNF assay data. A: BDNF levels of FSL rats after being left unstressed or exposed to the TDS paradigm and subsequently receiving sub-chronic VEH treatment. **B**: BDNF levels of FSL rats after being exposed to the TDS paradigm and subsequently receiving sub-chronic VEH or drug treatment, as indicated. All group sizes are equal (n = 12). Data points represent the mean ± SEM. Statistical analyses are reported in the text with ### p < 0,001 and d vs the TDS-naïve + VEH treatment group in **A**, and * p < 0,05, ** p < 0,01 and d vs the TDS-exposed + VEH treatment group in **B**. BDNF: brain-derived neurotrophic factor. d: unbiased Cohen's d-value. IMI-15: imipramine hydrochloride (15 mg/kg/day). SIL-3: sildenafil citrate (3 mg/kg/day). SIL-10: sildenafil citrate (10 mg/kg/day). TDS: time-dependent sensitisation. VEH: vehicle control.

Figure 4A). An Ordinary one-way ANOVA test for multiple comparisons in TDS-exposed FSL rats indicates statistically significant differences between the various sub-chronic treatment groups regarding hippocampal BDNF levels ($F_{(4, 55)}$ = 4,015, p = 0,0063). Dunnett's post-hoc test for multiple comparisons showed, in TDS-exposed FSL rats, that sub-chronic IMI-15 (p = 0,0317, d = 0,85), SIL-10 (p = 0,0078, d = 1,37) and IMI-15 + SIL-3 (p = 0,0024, d = 1,74) treatments significantly reduce hippocampal BDNF levels compared to VEH (see Figure 4B).

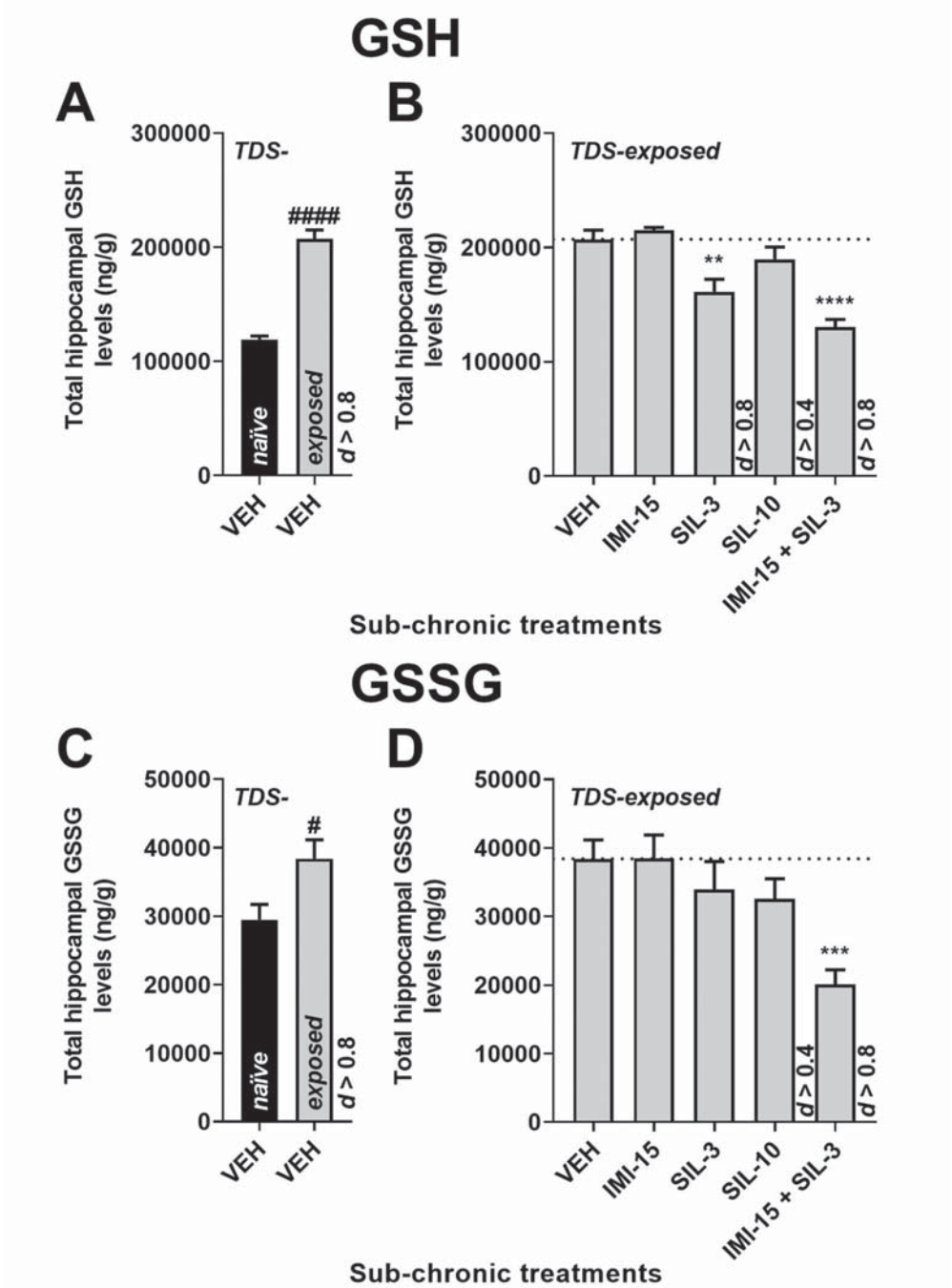

Figure 5: A graphical representation of the hippocampal GSH and GSSG assay data. A: GSH levels of FSL rats after being left unstressed or exposed to the TDS paradigm and subsequently receiving sub-chronic VEH treatment. **B**: GSH levels of FSL rats after being exposed to the TDS paradigm and subsequently receiving sub-chronic VEH or drug treatment, as indicated. **C**: GSSG levels of FSL rats after being left unstressed or exposed to the TDS paradigm and subsequently receiving sub-chronic VEH treatment. **D**: GSSG levels of FSL rats after being exposed to the TDS paradigm and subsequently receiving sub-chronic VEH or drug treatment, as indicated. All group sizes are equal (n = 12). Data points represent the mean ± SEM. Statistical analyses are reported in the text with #### p < 0,0001 and d vs the TDS-naïve + VEH treatment group in **A**, ** p < 0,01, **** p < 0,0001 and d vs the TDS-exposed + VEH treatment group in **B**, # p < 0,05 and d vs the TDS-naïve + VEH treatment group in **C**, *** p < 0,001 and d vs the TDS-exposed + VEH treatment group in **D**. d: unbiased Cohen's d-value. GSH: glutathione. GSSG: glutathione disulphate. IMI-15: imipramine hydrochloride (15 mg/kg/day). SIL-3: sildenafil citrate (3 mg/kg/day). SIL-10: sildenafil citrate (10 mg/kg/day). TDS: time-dependent sensitisation. VEH: vehicle control.

Hippocampal glutathione (GSH) and glutathione disulphide (GSSG) levels

Figure 5 depicts the total hippocampal GSH and GSSG levels of FSL rats after they were left unstressed or exposed to the TDS paradigm and subsequently received sub-chronic VEH or drug treatment.

An unpaired Student's t-test revealed that TDS exposure significantly elevates the hippocampal GSH levels of FSL rats compared to non-stressed FSL rats ($p < 0{,}0001$, $d = 4{,}17$) (see Figure 5A). An Ordinary one-way ANOVA test for multiple comparisons in TDS-exposed FSL rats indicates statistically significant differences between the various sub-chronic treatment groups regarding hippocampal GSH levels ($F_{(4, 52)} = 15{,}92$, $p < 0{,}0001$). Dunnett's post-hoc test for multiple comparisons showed, in TDS-exposed FSL rats, that sub-chronic SIL-3 ($p = 0{,}0018$, $d = 1{,}33$) and IMI-15 + SIL-3 ($p < 0{,}0001$, $d = 2{,}93$) treatments significantly reduces hippocampal GSH levels compared to VEH (see Figure 5B).

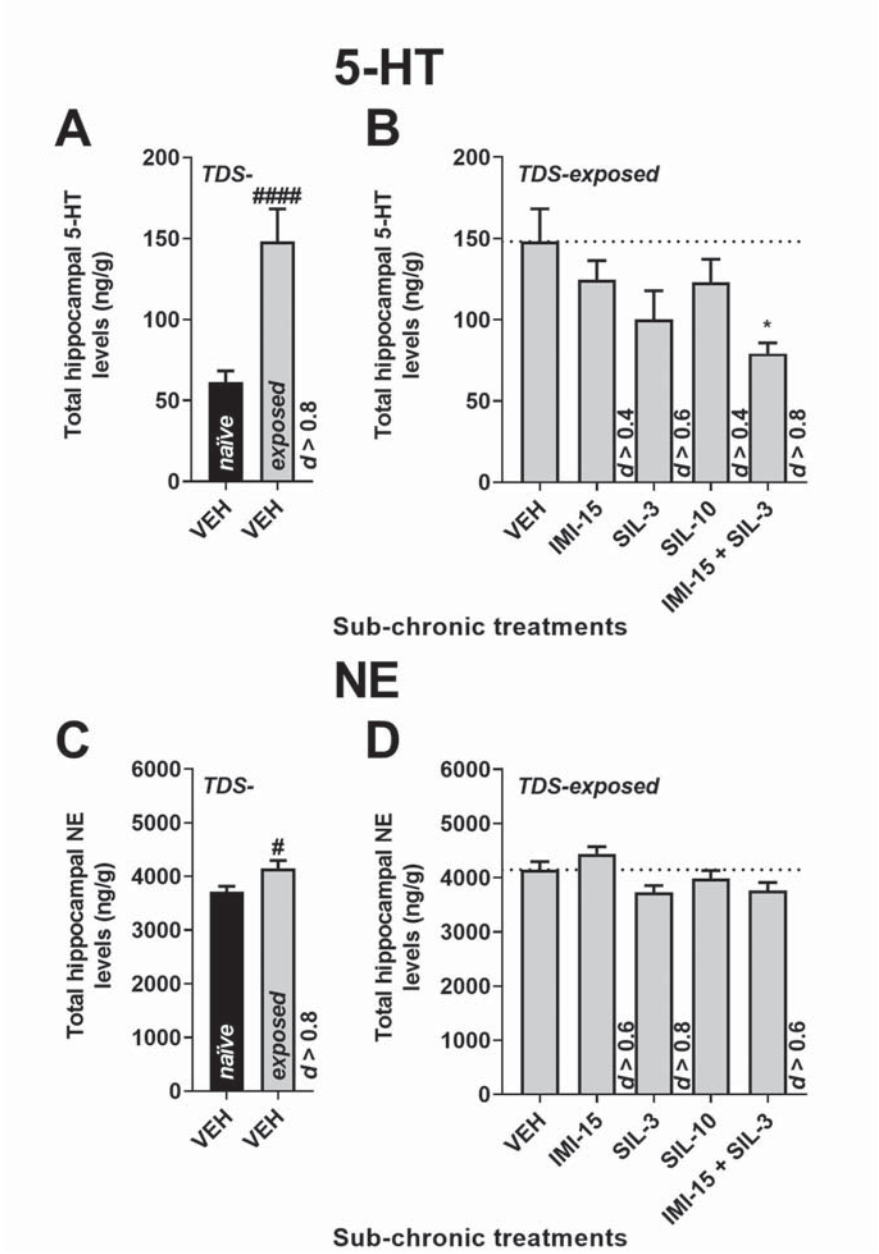

Figure 6: A graphical representation of the hippocampal 5-HT and NE assay data. A 5-HT levels of FSL rats after being left unstressed or exposed to the TDS paradigm and subsequently receiving sub-chronic VEH treatment. **B** 5-HT levels of FSL rats after being exposed to the TDS paradigm and subsequently receiving sub-chronic VEH or drug treatment, as indicated. **C** NE levels of FSL rats after being left unstressed or exposed to the TDS paradigm and subsequently receiving sub-chronic VEH treatment. **D** NE levels of FSL rats after being exposed to the TDS paradigm and subsequently receiving sub-chronic VEH or drug treatment, as indicated. All group sizes are equal (n = 12). Data points represent the mean ± SEM. Statistical analyses are reported in the text with #### $p < 0{,}0001$ and d vs the TDS-naïve + VEH treatment group in **A**, * $p < 0{,}05$ and d vs the TDS-exposed + VEH treatment group in **B**, # $p < 0{,}05$ and d vs the TDS-naïve + VEH treatment group in **C**, and d vs the TDS-exposed + VEH treatment group in **D**. d: unbiased Cohen's d-value. 5-HT: serotonin. IMI-15: imipramine hydrochloride (15 mg/kg/day). NE: norepinephrine. SIL-3: sildenafil citrate (3 mg/kg/day). SIL-10: sildenafil citrate (10 mg/kg/day). TDS: time-dependent sensitisation. VEH: vehicle control.

An unpaired Student's t-test revealed that TDS exposure significantly elevates the hippocampal GSSG levels of FSL rats compared to non-stressed FSL rats (p = 0,0185, d = 1,03) (see Figure 5C). A Kruskal-Wallis one-way ANOVA test for multiple comparisons in TDS-exposed FSL rats indicates statistically significant differences between the various sub-chronic treatment groups regarding hippocampal GSSG levels (p = 0,0004). Dunn's post-hoc test for multiple comparisons showed, in TDS-exposed FSL rats, that sub-chronic IMI-15 + SIL-3 (p = 0,0002, d = 2,15) treatment significantly reduces hippocampal GSSG levels compared to VEH-treated TDS-exposed FSL rats (see Figure 5D).

Hippocampal serotonin (5-HT) and norepinephrine (NE) levels

Figure 6 depicts the total hippocampal 5-HT and NE levels of FSL rats after they were left unstressed or exposed to the TDS paradigm and subsequently received sub-chronic VEH or drug treatment.

A Mann-Whitney u-test revealed that TDS exposure significantly elevates the hippocampal 5-HT levels of FSL rats compared to non-stressed FSL rats (p < 0,0001, d = 1,70) (see Figure 6A). A Kruskal-Wallis one-way ANOVA test for multiple comparisons in TDS-exposed FSL rats indicates statistically significant differences between the various sub-chronic treatment groups regarding hippocampal 5-HT levels (p = 0,0141). Dunn's post-hoc test for multiple comparisons showed, in TDS-exposed FSL rats, that sub-chronic IMI-15 + SIL-3 (p = 0,0115, d = 1,34) treatment significantly reduces hippocampal 5-HT levels compared to VEH (see Figure 6B).

An unpaired Student's t-test revealed that TDS exposure significantly elevates the hippocampal NE levels of FSL rats compared to non-stressed FSL rats (p = 0,0227, d = 0,99) (see Figure 6C). An Ordinary one-way ANOVA test for multiple comparisons in TDS-exposed FSL rats indicates statistically significant differences between the various sub-chronic treatment groups regarding hippocampal NE levels ($F_{(4, 54)}$ = 4,431, p = 0,0036). Dunnett's post-hoc test for multiple comparisons showed, in TDS-exposed FSL rats, that none of the sub-chronic drug treatments significantly altered hippocampal NE levels compared to VEH-treated TDS-exposed FSL rats (see Figure 6D).

Discussion

The key findings of this study are that TDS-exposed FSL rats display depressive-like behaviour greater than that in stress-naïve FSL rats, accompanied with significant hippocampal oxidative stress. More importantly, said depressive-like behaviour is associated with *elevated* hippocampal monoamines and *elevated* BDNF. Interestingly, IMI-15, SIL-10 and IMI-15 + SIL-3 reversed both the elevated BDNF and depressive-like behaviour in TDS + FSL rats but had varying effects on monoamine and redox changes. Moreover, SIL-3 augmented the antidepressant-like effects of IMI-15 in TDS-exposed FSL rats, while low dose SIL-3 on its own had negligible effects.

TDS exposure significantly elevated the general locomotor activity in FSL rats compared to TDS-naïve animals (see Figure 3A). This is, however, different from a similar study where locomotor activity was unchanged by TDS exposure (Brand & Harvey, 2017). While there are conflicting reports regarding the effect of imipramine on the locomotor activity in rats (Garcia et al., 2008; Liebenberg et al., 2010b; Réus et al., 2011; Guan et al., 2014; Brand & Harvey, 2017), here IMI-15 significantly reduced general locomotor activity in TDS-exposed rats (see Figure 3B). This is not so unexpected considering the sedative effects of imipramine (Rickels et al., 1993; Ramirez & Sheridan, 2016), although this has often been described in rodents, and has been associated with long-term neurobiological receptor changes (Aulakh et al., 1987), but not fully elucidated to the best of our knowledge. Sub-chronic SIL-10 but not SIL-3 treatment also significantly reduced the general locomotor activity of TDS-exposed rats compared to controls (see Figure 3B). While previous studies, albeit without TDS exposure, did not find any altered locomotor activity in rats following SIL-3 and SIL-10 treatments (Liebenberg et al., 2010a; Liebenberg et al., 2010b; Saayman et al., 2021), the response as noted here is very likely related to the anxiogenic characteristics of TDS and that PDE5 inhibitors also have anxiolytic actions in FSL rats (Liebenberg et al., 2012), as working hypothesis for prospective studies in models of TRD. Likewise, sub-chronic IMI-15 + SIL-3 treatment also significantly reduced general locomotor activity in TDS-exposed rats, as compared to VEH controls (see Figure 3B).

Considering that TDS and the drugs used in this study significantly alter general locomotor activity, the FST data were subsequently corrected to offset the influence of this covariable to reflect psychomotor activity more specifically (i.e., depressive-like behaviour). To this end ANCOVA adjustments were applied (Steyn et al., 2018), as depicted in Figure 3C and Figure 3D. TDS exposure significantly elevated the depressive-like behaviour (time spent immobile) of FSL rats compared to TDS-naïve FSL rats (see Figure 3C). This is consistent with earlier findings (Brand & Harvey, 2017) and confirm that an exaggerated depressive-like phenotype is a primary feature of the TDS + FSL model of TRD, suggesting robust face validity. However, sub-chronic IMI-15 treatment significantly reduced the depressive-like behaviour of TDS-exposed FSL rats compared to control FSL rats, albeit with medium effect size (see Figure 3D).

Thus, treatment resistance in this model was not robust. That said, Brand and Harvey (2017) showed that imipramine treatment significantly reduced depressive-like behaviour in both TDS-naïve and TDS-exposed FSL rats, but that depressive-like behaviour remained significantly greater in TDS-exposed versus TDS-naïve FSL rats (Brand & Harvey, 2017). In fact, MDD has often been described as a heterogenous disorder, so that TRD, a more severe manifestation thereof, would likely be even more complex and multifactorial in both its aetiology and underlying neurobiological basis. In this regard then stands the current TDS + FSL model, an aetiological model based on the development of depressive-like behaviour following three acute traumatic experiences plus contextual reminders (i.e., causality), which may also correlate aetiologically with

posttraumatic development of TRD in humans. As such the animal model is expected to model not only TRD, but also elements of other common co-morbidities, such as anxiety, which is typical of posttraumatic stress disorder (Brand & Harvey, 2017). Consequently, drug treatment responses may also not be as straightforward, predictable or robust as in a more simplistic TRD model, as we have earlier presented using the adrenocorticotropic hormone (ACTH) model of TRD (Saayman et al., 2024). Despite these limitations, it does reflect a model that shows real life variability across animals and studies, not unlike the variable disease state that predetermines treatment response, or lack thereof, in patients with TRD (Nierenberg & DeCecco, 2001). Taken together, antidepressant treatment resistance may not be as profound in this model, as say post weaning social isolation rearing of FSL rats (Mncube et al., 2021; Mncube & Harvey, 2022).

Several studies have demonstrated the antidepressant-like properties of PDE5 inhibitors in treatment-responsive rodent models of MDD (Brink et al., 2008; Liebenberg et al., 2010a; Liebenberg et al., 2010b; Matsushita et al., 2012; Tomaz et al., 2014; Wang et al., 2014; Socała et al., 2016; Saayman et al., 2021). Here we show that sub-chronic SIL-10 treatment alone significantly reduced depressive-like behaviour in TDS-exposed FSL rats compared to FSL controls (with large effect size), whereas sub-chronic SIL-3 treatment showed a similar trend of medium effect size (see Figure 3D). One can therefore argue that the TDS + FSL model of TRD responds to sub-chronic SIL-3 and SIL-10 treatments. Thus, PDE5 inhibitors (such as sildenafil) induce dose-dependent antidepressant-like effects. Recently, we showed the same dose-dependent antidepressant-like activity for sildenafil in an adrenocorticotropic hormone-induced (ACTH) rodent model of TRD (Saayman et al., 2024).

Moreover, sub-chronic IMI-15 + SIL-3 treatment significantly reduced the depressive-like behaviour of TDS-exposed rats compared to FSL controls, supported by a large effect size (see Figure 3D). In fact, SIL-3 augmented the antidepressant-like effects of IMI-15 in TDS-exposed FSL rats (see Figure 3D). It has been demonstrated previously that higher doses of sildenafil in rats may require concurrent antimuscarinic activity (such as that provided by imipramine in this study) to unmask its antidepressant-like effects (Brink et al., 2008; Liebenberg et al., 2010a). This may explain the findings of the current study. Taken together, sildenafil, may be able to exert antidepressant-like effects in the TDS-induced rodent model of TRD.

Like the ACTH-based model of TRD, where sub-chronic ACTH treatment elevates hippocampal BDNF (Saayman et al., 2024), TDS exposure significantly elevated hippocampal BDNF levels compared to TDS-naïve rats (see Figure 4A), while sub-chronic IMI-15 treatment significantly reduced this elevation in hippocampal BDNF levels in TDS-exposed FSL rats compared to FSL controls (see Figure 4B). Considering the neurotrophic hypothesis of MDD, where MDD is typically associated with reduced hippocampal BDNF levels (Jacobsen & Mørk, 2006; Huang et al., 2011; Antunes et al., 2015; Gong et al., 2016), these data are paradoxical. Indeed, antidepressants (and non-pharmacological interventions) have been shown to increase BDNF levels (Balu et al., 2008; Vidal et al., 2011; Sheldrick et al., 2017; Mondal & Fatima, 2019). The latter is also associated with a reversal of depressive symptoms (Duman, 2002; Duman & Monteggia, 2006; Piccinni et al., 2015; Polyakova et al., 2015; Duman et al., 2016). As with IMI-15, sub-chronic SIL-10 and IMI-15 + SIL-3 treatments also significantly reduced hippocampal BDNF levels in TDS + FSL rats, with SIL-3 alone inducing a large effect size decrease in BDNF (see Figure 4B). In fact, we have demonstrated a similar antidepressant-induced reduction of BDNF concentrations in an adrenocorticotropic hormone (ACTH)induced model of TRD in Sprague Dawley rats (Saayman et al., 2024).

Taken together, TDS + FSL rats present with elevated hippocampal BDNF and depressive-like behaviour, a paradoxical finding. Nevertheless, that both were effectively reversed by an antidepressant is a noteworthy finding. By adversely affecting resilience, BDNF facilitates activity-dependent plasticity that may translate to a variable effect on mood and other plasticity-dependent functions. Thus, Martinez and colleagues (2012) found a positive correlation between BDNF and severity of suicidal ideation (Martinez et al., 2012), while another clinical study describes worsening redox and metabolic status as a possible counterregulatory action of BDNF (Harvey et al., 2012). Thus, depending on illness state or the persistence of an adverse environment, BDNF may mediate undesirable redox and metabolic changes that promote the development of depression (Harvey et al., 2012). In our study, this could represent the combined effect of TDS plus the FSL rat strain. In support of this, direct injection of BDNF into mesolimbic dopamine (DA) circuits exerts a paradoxical depressogenic effect (Castrén et al., 2007). So, while the antidepressant-like effects of sildenafil are associated with the elevation of cyclic guanosine monophosphate (cGMP)/BDNF signalling in the hippocampus (Wang et al., 2014), the presence of severe adversity may have counterregulatory actions producing the results shown here. The question is whether any undesirable redox and neurochemical changes were evident in TDS + FSL rats, and how these responded to treatment.

TDS + FSL rats presented with significantly elevated hippocampal GSH and GSSG levels compared to TDS-naïve animals (see Figure 5A and Figure 5C, respectively). Considering that MDD and TRD are associated with elevated oxidative stress and reduced levels of GSH as antioxidant (Lapidus et al., 2014; Black et al., 2015; D Rosenblat et al., 2015; Liu et al., 2015), one needs to bear in mind that this is not MDD or TRD but an animal model of these conditions. Therefore, the results may not parallel the clinical condition exactly, yet still argue strongly for a state of redox imbalance in TDS + FSL rats. In fact, the ACTH-based TRD model noted above also displays increased GSH (Saayman et al., 2024). That said, low intracellular GSH levels decrease cellular antioxidant capacity, whereas elevated GSH levels usually increase antioxidant capacity and resistance to oxidative stress (Ballatori et al., 2009). Inactivation of reactive oxygen species (ROS) involves oxidation of GSH into GSSG (Prchal et al., 1975; Griffith, 1999). Thus, the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) may be used as a marker of oxidative stress (Zitka et al., 2012). This would concur with the arguments made

above that redox disturbances may co-present with elevated BDNF.

Sub-chronic IMI-15 treatment did not significantly affect hippocampal GSH and GSSG levels in TDS-exposed FSL rats compared to FSL controls (see Figure 5B and Figure 5D, respectively). Thus, at the simplest level the antidepressant effects of IMI-15 are not redox dependent. However, sub-chronic SIL-3, and IMI-15 + SIL-3 treatments significantly reduced GSH levels in TDS + FSL rats, confirming the antioxidant action of PDE5 inhibitors (Swiecicka, 2023). Interestingly, this response seems to be dose-dependent since sub-chronic SIL-10 treatment did not alter GSH levels (see Figure 5B). This is not unlike its antidepressant-like actions noted earlier. That said, only sub-chronic IMI-15 + SIL-3 treatment significantly reduced the hippocampal GSSG levels of TDS-exposed rats compared to controls (see Figure 5D).

TDS exposed FSL rats showed significantly elevated hippocampal 5-HT and NE levels compared to TDS-naïve animals (see Figure 6A and Figure 6C, respectively), in line with a previous study in mice following psychological stress (MA et al., 2008). Moreover, another TRD model has also shown elevated hippocampal 5-HT (Saayman et al., 2024). In fact, elevated 5-HT has been shown to have depressogenic actions under certain conditions (Andrews et al., 2015). Contrary to its mode of action, sub-chronic IMI-15 treatment did not significantly alter hippocampal 5-HT and NE levels in TDS + FSL rats compared to FSL controls (see Figure 6B and Figure 6D, respectively), although a medium effect size increase in NE was evident as predicted (see Figure 6D) (Taylor et al., 2005; Sheffler et al., 2019). Sub-chronic SIL-3 and SIL-10 treatments also did not significantly alter hippocampal 5-HT and NE levels in TDS-exposed FSL rats compared to FSL controls. However, sub-chronic SIL-3 treatment displayed a medium and large effect size trend towards lowering hippocampal 5-HT and NE, respectively (see Figure 6B and Figure 6D). Sub-chronic IMI-15 + SIL-3 treatment significantly reduced hippocampal 5-HT levels, with a medium effect size trend towards lowering hippocampal NE levels (see Figure 6B and Figure 6D, respectively).

By virtue of these findings, the neurobiological construct of TRD may differ from that of MDD, and hence invariably requires different pharmacological approaches. For one, TRD models seem to share elements of oxidative stress, which is known to exert diverse effects on regional brain monoamines (Möller et al., 2013). More relevant, the presence of oxidative stress, such as ozone exposure, inhibits the antidepressant actions of imipramine (Mokoena et al., 2010; Mokoena et al., 2015). It would appear that the TDS-based rodent model of TRD presents with elevated hippocampal BDNF levels, which have also been demonstrated in another TRD model (Saayman et al., 2024), whereas the opposite is evident in rodent models of MDD, such as the FSL rat (Elfving et al., 2010). Importantly, elevated hippocampal 5-HT and NE levels have been demonstrated in the TDS + FSL (this work) and ACTH models of TRD (Saayman et al., 2024), as well as FSL rats (Zangen et al., 1997; Zangen et al., 1999).

As such, elevated hippocampal 5-HT and NE levels seem to be a consistent pathophysiological feature of both MDD and TRD, at least in rodents. This seems paradoxical, although 5-HT is known to be a Janus-faced molecule, having both beneficial and deleterious effects on cellular and behavioural function (Harvey et al., 2004; Andrews et al., 2015). The TDS + FSL model also presents a construct of anxiety that, in the context of TRD, is important, given that humans with post-traumatic stress disorder (PTSD) invariably develop TRD (Brand & Harvey, 2017). This also needs consideration in interpreting the response to treatment, as has been noted earlier. A significant limitation to this study needs to be mentioned. Despite some evidence for treatment resistance, sub-chronic IMI-15 (traditional monoaminergic antidepressant) treatment induced antidepressant-like effects in the TDS model.

TDS-exposed FSL rats displayed depressive-like behaviour and evidence for oxidative stress compared to TDS-naïve FSL rats. Paradoxically, TDS-exposed FSL rats also presented with elevated hippocampal monoaminergic and BDNF levels, suggesting a counterregulatory action of BDNF, as well as elevated motor function. However, the exact biochemical mechanisms whereby sub-chronic IMI-15, SIL-10 and IMI-15 + SIL-3 treatments produce their antidepressant-like effects in the TDS model of TRD remain unclear. The contributions made by this study to TRD research may be seminal and warrant further investigation, particularly considering the significant challenges currently associated with the treatment of the disorder.

Ethical standards

All attempts were made to curtail the suffering of animals during this study. All ethical considerations were deliberated on independently, a proper harm-benefit analysis was performed, and all possible mitigating factors were implemented to ensure that this severely impactful procedure on rodents could be justified. Keeping in mind respect for sentient animals, and the severe suffering of humans with TRD, as well as understanding the ethical dilemma posed, the study was considered ethically justified under strict conditions.

The Animal Care, Health, and Safety Research Ethics Committee (NHREC reg. no.: AREC-130913-015) of the Faculty of Health Sciences, NWU, South Africa, approved this study (NWU-AnimCareREC ethics approval no.: NWU-00598-19-A5). All experiments and procedures performed on animals as part of this study and their housing conditions complied with the institutional policies and other guidelines on the care and use of laboratory animals, as well as national legislation. The way experiments and procedures were carried out and how animals were kept were also informed by the guidelines of the South African National Standards: The Care and Use of Animals for Scientific Purposes (SANS 10386:2008), as well as the Ethics in Health Research: Principles, Processes and Structures guidelines of 2015.

To improve the conducting, reporting and appraisal of animal research, Smith and colleagues (2018) have recently developed a set of planning guidelines known as the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines (Smith et al., 2018). In this regard, the PREPARE guidelines were meticulously considered and implemented during the planning phase of this study to improve the quality, reproducibility, and translatability of experimental results. Moreover, to promote a transparent, reproducible, accurate, comprehensive, concise, logically ordered, and well-written manuscript, all experimental data are reported according to the National Centre for the Replacement, Refinement and Reduction of Animals in Research's (NC3Rs) Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (Kilkenny et al., 2010).

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