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Combined administration of scopolamine and a negative allosteric modulator of the metabotropic glutamate mGlu2 receptor as a novel efficacious method to treat depression

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Połączone podawanie skopolaminy i negatywnego modulatora allosterycznego metabotropowego receptora glutaminergicznego mGlu2 jako nowa skuteczna metoda leczenia depresji

Streszczenie

Zaburzenia depresyjne są powszechnie uznawane za jedne z najczęstszych i najbardziej wyniszczających zaburzeń psychicznych. Pomimo dostępności licznych konwencjonalnych leków przeciwdepresyjnych, głównie oddziałujących na układ monoaminergiczny, ich skuteczność pozostawia wiele do życzenia — często przynoszą one opóźnioną i jedynie częściową ulgę. W związku z tym potrzeba skuteczniejszych i szybko działających leków jest wciąż aktualna i niezwykle istotna.

Liczne badania kliniczne i przedkliniczne wskazują, że skopolamina — nieselektywny antagonista cholinergicznych receptorów muskarynowych — wywiera szybkie i długotrwałe działanie przeciwdepresyjne. Niemniej jednak jej zastosowanie kliniczne jest ograniczone z uwagi na istotne działania niepożądane, takie jak zaburzenia pamięci, sedacja oraz zaburzenia wzrokowe. Jedną z potencjalnych strategii zmniejszenia działań niepożądanych skopolaminy jest jej współpodawanie w niskich dawkach z innymi substancjami o właściwościach przeciwdepresyjnych. Na takie podejście terapeutyczne zdecydowano się w ramach niniejszej pracy. Podprogowe dawki (niewykazujące działania podobnego do przeciwdepresyjnego) skopolaminy połączono z podprogowymi dawkami VU6001966 — negatywnego modulatora allosterycznego (NAM) receptora mGlu2.

Wszystkie eksperymenty zostały przeprowadzone na samcach myszy szczepu C57BL/6J lub szczurach szczepu Sprague Dawley. Aby ocenić przeciwdepresyjne działanie testowanej kombinacji substancji, myszy poddano modelowi nieprzewidywalnego, przewlekłego i łagodnego stresu (Unpredictable Chronic Mild Stress, UCMS), będącemu uznanym modelem depresji opartym na chronicznym stresie. Model ten pozwala na ocenę parametrów odzwierciedlających kluczowe objawy depresji oraz odróżnienie klasycznych leków przeciwdepresyjnych od substancji o szybkim działaniu. Do analizowanych parametrów behawioralnych należały: skrócony czas pielęgnacji w teście pielęgnacyjnym (Splash Test), interpretowany jako przejaw apatii, zmniejszona preferencja spożycia sacharozy w teście preferencji sacharozy (Sucrose Preference Test, SPT), będąca wskaźnikiem anhedonii, oraz wydłużony czas bezruchu w teście zawieszenia za ogon (Tail Suspension Test, TST) i w

teście wymuszonego pływania (Forced Swim Test, FST), odzwierciedlający behawioralny odpowiednik bezradności.

Aby zbadać mechanizmy leżące u podstaw zaobserwowanych efektów, użyto antagonistów receptorów AMPA (NBQX) oraz TrkB (ANA-12), w celu określenia ich roli w działaniu podobnym do przeciwdepresyjnego badanych związków. Dodatkowo zbadano rolę szlaków sygnalizacyjnych mTOR oraz BDNF/TrkB poprzez określenie poziomów wybranych białek za pomocą techniki Western Blot.

Aby ocenić ryzyko potencjalnych działań niepożądanych badanych związków, przeprowadzono test aktywności lokomotorycznej w celu oceny ogólnej aktywności, a także test lokalizacji obiektu (Object Location Test, OLT) i test rozpoznawania nowego obiektu (Novel Object Recognition Test, NORT) w celu oceny ich wpływu na pamięć.

Ponadto, za pomocą techniki mikrodializy u swobodnie poruszających się szczurów zbadano wpływ badanych związków na zewnątrzkomórkowy poziom kluczowych neuroprzekaźników, takich jak serotonina, dopamina, glutaminian i GABA, w korze przedczołowej (FCX) szczura.

Uzyskane wyniki potwierdzają, że dołączenie VU6001966 do skopolaminy nie tylko zwiększa jej skuteczność przeciwdepresyjną, ale może również łagodzić działania niepożądane typowe dla skopolaminy. Kombinacja ta, podawana przez cztery kolejne dni, nie wpływała na aktywność lokomotoryczną ani pamięć przestrzenną. Ponadto, ostre efekty przeciwdepresyjne były związane ze wzrostem zewnątrzkomórkowych poziomów glutaminianu, dopaminy i serotoniny w korze przedczołowej, natomiast utrzymujące się efekty po leczeniu subchronicznym wydają się zależeć od aktywacji receptorów AMPA i TrkB.

Podsumowując, połączone podawanie skopolaminy z VU6001966 — negatywnym modulatorem allosterycznym receptora mGlu2 — może mieć stotny potencjał kliniczny, pozwalając na stosowanie niższych dawek terapeutycznych skopolaminy przy jednoczesnym zachowaniu funkcji poznawczych. Należy jednak uwzględnić pewne ograniczenia — ze względu na możliwe różnice międzygatunkowe oraz wpływ płci, wyników badań przedklinicznych nie można bezpośrednio ekstrapolować na depresję u ludzi.

Combined administration of scopolamine and a negative allosteric modulator of the metabotropic glutamate mGlu2 receptor as a novel efficacious method to treat depression

Abstract

Major depressive disorder (MDD) is widely recognized as one of the most prevalent and debilitating mental health problems. Despite the availability of numerous conventional antidepressants primarily targeting the monoaminergic system, they are far from ideal and often produce delayed and only partial relief. Consequently, the need for more effective and rapid-acting compounds is as timely and compelling as ever.

Numerous clinical and preclinical studies indicate that scopolamine, a non-selective muscarinic cholinergic receptor antagonist, exerts rapid and long-lasting antidepressant effects. However, its clinical use is limited by considerable adverse effects, including memory impairment, sedation, and visual disturbances. One potential strategy to mitigate these adverse effects is the coadministration of scopolamine at low doses with other compounds that possess antidepressant properties. This therapeutic approach was implemented in the present doctoral thesis, wherein subeffective doses of scopolamine were combined with subeffective doses of the mGlu2 negative allosteric modulator (NAM) VU6001966.

All experiments were conducted on male C57BL/6J mice or Sprague Dawley rats. To evaluate the antidepressant-like effects of the tested combination, a chronic stress-based mouse model of depression — the unpredictable chronic mild stress (UCMS) paradigm — was employed. This model enables the assessment of parameters that reflect core depressive symptoms and the distinction between classical and rapid-acting antidepressants. These behavioral parameters included reduced grooming time in the splash test, indicating apathy; decreased sucrose preference in the sucrose preference test (SPT), serving as a measure of anhedonia; and increased immobility in the tail suspension test (TST) and the forced swim test (FST), reflecting behavioral despair.

To explore the mechanisms underlying the observed effects, the AMPA receptor antagonist NBQX and TrkB receptor antagonist ANA-12 were used to determine the involvement of these receptors in antidepressant-like action. Additionally, the role of mTOR and BDNF/TrkB signaling pathways was investigated by measuring the expression levels of selected proteins using the Western Blot technique.

To evaluate the risk of potential adverse effects from the tested combination, we conducted a locomotor activity test to assess general activity, as well as object location (OLT) and novel object recognition (NORT) tests to assess memory.

Moreover, microdialysis in freely moving rats was used to examine the effects of the compounds on extracellular levels of key neurotransmitters (serotonin, dopamine, glutamate, and GABA) in the rat frontal cortex (FCX).

The results support the notion that combining scopolamine with the mGlu2 NAM VU6001966 not only enhances its antidepressant efficacy but may also attenuate adverse effects commonly associated with scopolamine use. Subchronic coadministration of scopolamine and VU6001966 over four consecutive days did not impair locomotor activity or spatial and non-spatial memory. Furthermore, the acute antidepressant-like effects were associated with increased extracellular levels of glutamate, dopamine, and serotonin in the frontal cortex, whereas sustained effects following subchronic treatment appeared to depend on AMPA and TrkB receptor activation.

In summary, the coadministration of scopolamine with the mGlu2 NAM VU6001966 may offer significant clinical potential by allowing lower therapeutic doses of scopolamine while preserving cognitive function. Nonetheless, some limitations must be acknowledged. Given the possibility of interspecies and sex differences, findings from these preclinical studies should not be directly extrapolated to the human condition of depression.

Abbreviations

Abbreviation	Description
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	brain-derived neurotrophic factor
DA	dopamine
eEF2	eukaryotic elongation factor 2
FST	forced swim test
GABA	γ-aminobutyric acid
GLU	glutamate
MDD	major depressive disorder
mTOR	mammalian target of rapamycin
NAM	negative allosteric modulator
NORT	novel object recognition test
OLT	object location test
PFC	prefrontal cortex
PSD95	postsynaptic density protein 95
SPT	sucrose preference test
TrkB	tropomyosin receptor kinase B
TST	tail suspension test
UCMS	unpredictable chronic mild stress

This thesis is based on the following publications of the author:

[1] **Babii, Y.**, Pałucha-Poniewiera, A., Rafało-Ulińska, A., Brański, P., Pilc, A. (2024). Subchronic administration of scopolamine reverses UCMS-induced behavior in mice via eEF2 protein dephosphorylation. *Pharmacological Reports, 76*(5), 1001–1011. **DOI:** 10.1007/s43440-024-00630-4.
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[3] **Babii, Y.**, Pałucha-Poniewiera, A., Bobula, B., Kania, A., Bederska-Łojewska, D., Brański, P., Pilc, A. Coadministration of Scopolamine and mGlu2 NAM VU6001966 as a Novel Antidepressant Approach: Lowering Effective Dose and Reducing Cognitive Side Effects. *Psychopharmacology* — Accepted for publication.
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"All things are poison, and nothing is without poison;
only the dose makes a thing not a poison."

—Paracelsus, 1538

Contents

Chapter 1

Introduction

1.1 Depression

Major depressive disorder (MDD) is a severe and recurrent disease that considerably affects those who suffer from it and imposes a heavy burden on society. Its alarming rise became especially prominent during the 20th century. The global prevalence of depression has increased by nearly 50% over the past 30 years (Liu et al., 2020), with approximately 332 million people of all ages currently affected (World Health Organization, 2023). By 2030, depression is anticipated to be the leading cause of disease burden worldwide, according to WHO estimates (World Health Organization, 2011). Recurrences across the lifespan following an initial depressive episode are also a significant concern, as approximately 75–90% of individuals with MDD will experience more than one episode (Monroe & Harkness, 2022). The discovery of conventional antidepressants marked a significant breakthrough in the treatment of depression. However, despite their acute effects on the monoaminergic system, currently available antidepressant drugs are far from ideal, typically producing a slow and often incomplete therapeutic response (Machado-Vieira et al., 2010; Parker et al., 2001). Only about 30% of patients with MDD achieve full remission following adequate treatment (Gaynes et al., 2020), while a similar proportion meet the criteria for treatment-resistant depression (TRD), defined as an inadequate response to at least two antidepressants from different classes, each given at an optimal dose and for an adequate duration (Jaffe et al., 2019). Given these alarming statistics, understanding the mechanisms underlying depression remains one of the most crucial challenges in modern neuroscience. Additional strategies are needed to address the problem and alleviate the

patient's depressive symptoms.

The first mentions of symptoms associated with depression were documented thousands of years ago (Reynolds & Wilson, 2013). Such symptoms were originally conceptualized as melancholia, explained by the humoral theory of causation as a result of an excess of black bile. Melancholia was regarded primarily as a disorder of the intellect, with sadness present but not considered its defining feature (Kendler, 2020). It was not until the late 18th century that the modern view of depression as a mood disorder began to emerge (Kendler, 2020). The term depression subsequently came into clinical use in the 19th century, initially referred to as "mental depression" (Paykel, 2008).

As knowledge advanced, the diagnostic system evolved into its current form, the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5). According to the DSM-5, a diagnosis of MDD requires at least five symptoms — one of which must be either a depressed mood or a loss of interest or pleasure (anhedonia) — present during the same two-week period. Other core symptoms include persistent ruminations, feelings of worthlessness or excessive guilt, fatigue, sleep and psychomotor disturbances, cognitive impairment, and, in severe cases, suicidal ideation. The DSM-5 reflects the heterogeneity of MDD, resulting in a possible 227 different symptom combinations that meet diagnostic criteria.

To assess both the severity of the illness and the patient's response to antidepressant therapy, several standardized tools can be used, including the Beck Depression Inventory II (BDI-II), the Hamilton Rating Scale for Depression (HRSD), the Montgomery Asberg Depression Rating Scale (MADRS), and the Snaith-Hamilton Pleasure Rating Scale (SHAPS) (E. D. Ballard et al., 2018).

Depression is highly heterogeneous in both its causes and underlying biological mechanisms. Several factors are theorized to contribute to depression, including neurotransmitter dysfunction, hypothalamic-pituitary-adrenal (HPA) axis disturbances, chronic low-grade inflammation, mitochondrial dysfunction and oxidative damage, gut microbial dysbiosis, and liver dysfunction (Cui et al., 2024; Larrea et al., 2024). Structural imaging has consistently highlighted abnormalities in the prefrontal cortex (PFC) and hippocampus. Several studies revealed reduced volume and decreased activity in these regions (Arnone et al., 2012; Videbech & Ravnkilde, 2004), which are critical for memory consolidation, problem solving, sustaining attention, and emotional processing. Additionally, some stud-

ies report hypertrophy of the nucleus accumbens (NAc) and amygdala (Abdallah et al., 2017; McEwen et al., 2016), structures involved in motivation, emotion regulation, and stress response.

Given the high complexity of depression, identifying reliable biological markers applicable to most patients is challenging, contributing to underdiagnosis, particularly in the early stages of the disorder. By definition, an ideal biomarker should be quantifiable from an easily obtainable sample, which is why most studies focus on liquid biopsies such as blood. Some proteins have been reported in multiple studies as potential biomarkers for MDD. Notably, patients with MDD have been found to exhibit lower nocturnal melatonin levels (Buckley & Schatzberg, 2010) and reduced growth hormone secretion (Birmaher et al., 2000), as well as elevated inflammatory markers — primarily TNF-alpha, IL-6, and C-reactive protein (Dowlati et al., 2010; Howren et al., 2009) — in plasma, with the latter correlating with symptom severity (Aleem & Tohid, 2018). However, further studies are needed to confirm the applicability of these measures as reliable biomarkers.

1.2 Monoamine-Based Pharmacology of Depression

The discovery of the first antidepressant treatments dates back to the 1950s. Before then, opium was the primary pharmacological option for depression treatment. The advent of antidepressants began with the accidental discovery of monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs). Iproniazid, an MAOI initially used as an antituberculosis drug, was first reported by (Selikoff & Robitzek, 1952) to cause a "general stimulation" as a side effect. Its potential for treating depression was later evaluated by Kline and colleagues in depressed patients without tuberculosis (Loomer et al., 1957). Around the same time, imipramine, a tricyclic drug tested for its neuroleptic effects, was found to have antidepressant properties and was released in 1957 (Kuhn, 1958).

MAOIs prevent monoamine breakdown, leading to a broad and sustained increase in their levels, whereas TCAs block monoamine reuptake and additionally antagonize various receptors, resulting in a mixed pharmacological profile. Despite their numerous side effects, MAOIs and TCAs are still used with great success, especially in patients who are unresponsive to other treatments (Kim et al., 2019). The accidental discoveries of

these drugs marked a major milestone in understanding the pathophysiology of depression. They paved the way for the first neurobiological hypothesis of a psychiatric disorder, connecting mood regulation with chemical imbalances in the brain, mainly involving serotonin and noradrenaline (Lapin & Oxenkrug, 1969; Schildkraut, 1965).

In the late 1980s, selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) were introduced to the market. These drugs are now considered first-line medications for MDD because of their greater selectivity and higher safety profile, resulting in fewer adverse effects (Lane et al., 1995).

Conventional antidepressants primarily act by enhancing monoaminergic tone. In depressive patients, levels of 5-hydroxyindoleacetic acid (5-HIAA), a serotonin metabolite, in the cerebrospinal fluid (CSF), as well as urinary concentrations of serotonin and 5-HIAA, are often reduced (Van Praag et al., 1970). The serotonin hypothesis of depression has been influential for decades and serves as a foundation for the use of antidepressants. However, its validity and clinical relevance have been questioned, with some experts arguing that the mechanisms underlying depression are far more complex and multidimensional than the monoaminergic model suggests (Moncrieff et al., 2023). Recent studies have shown that antidepressant use can lead to a reduction in plasma serotonin levels, as well as lower levels of its metabolite 5-HIAA, in CSF (Huang et al., 2021; Pech et al., 2018). Animal studies involving chronic treatment with the SSRI citalopram also revealed decreased serotonin levels throughout the brain (Bosker et al., 2010). Furthermore, patients treated with MAOIs or SSRIs have been found to have higher relapse rates compared to those treated with noradrenergic tricyclics (73 vs. 18%) (Delgado et al., 1991).

These findings raise important questions about whether they confirm the ambiguity of the monoaminergic hypothesis of depression or simply reflect compensatory changes induced by long-term antidepressant use (Fava, 2020). Such insights may help explain why monoamine-based drugs are effective in treating acute depressive episodes, yet often fail in long-term maintenance therapy. The delayed, and in many cases insufficient, response to current antidepressants further suggests that mechanisms beyond those proposed by the monoamine theory may play an equally important role in the pathophysiology of depression. The need for novel therapeutic strategies thus remains both urgent and critical. Advancements in this area hold the promise of developing more effective treatments, eventually improving the quality of life for hundreds of millions worldwide.

1.3 The Cholinergic Component of Depression

The association between elevated acetylcholine (ACh) levels and depression was first observed in the 1950s when individuals exposed to irreversible cholinesterase inhibitors, such as insecticides or nerve agent weapons, developed psychiatric symptoms including depression (Gershon & Shaw, 1961; Rowntree et al., 1950). Twenty years later, Janowsky and colleagues introduced the adrenergic-cholinergic balance hypothesis of mania and depression, suggesting that increased cholinergic and decreased noradrenergic tone may underlie depression (Janowsky et al., 1972). Evidence supporting this hypothesis includes findings that acetylcholinesterase inhibitors (AChEIs), such as physostigmine, enhance cholinergic activity and induce depressive symptoms such as sedation, reduced speech, and decreased spontaneous activity in both healthy individuals (Risch et al., 1981) and manic patients (K. L. Davis et al., 1978), as well as worsen preexisting depression in patients (Modestin et al., 1973).

Further studies confirm the role of the cholinergic system in depression. It was shown that among individuals with major depression living in agricultural areas, there was a negative correlation between red blood cell acetylcholinesterase activity and both hopelessness levels and the number of prior suicide attempts (Altinyazar et al., 2016). Additionally, a single-photon emission computed tomography (SPECT) imaging study has revealed elevated cortical and subcortical ACh levels in actively depressed patients, persisting even in those who have recovered (Hannestad et al., 2013; Saricicek et al., 2012). Postmortem studies have demonstrated no significant differences in the availability of $\beta 2$ subunit-containing nicotinic acetylcholine receptors ($\beta 2*$-nAChR) between depressed patients and controls, suggesting that receptor availability changes are not due to a reduced total number of receptors. Furthermore, acetylcholine precursors such as choline (trimethylaminoethanol) or deanol (dimethylaminoethanol) have been reported to induce depressive symptoms in certain patients (Casey, 1979; Tamminga et al., 1976).

Similar to findings in humans, animal studies have shown that increased cholinergic tone can precipitate depressive-like behaviors. For example, both direct-acting muscarinic agonist arecoline and the indirect-acting AChEI physostigmine have been found to reduce the intracranial self-stimulation (ICSS), a measure of reward threshold (Olds & Domino, 1969), and increase immobility in the tail suspension test (TST) and forced

swim test (FST), indicating behavioral despair (Chau et al., 2001; Mineur et al., 2013; van Enkhuizen et al., 2015). The Flinders Sensitive Line (FSL), selectively bred from Sprague-Dawley rats for increased responses to anticholinesterase agents, displays depressive-like behavior such as reduced activity and appetite, impaired psychomotor function, and immune abnormalities — characteristics commonly observed in depressed individuals (Overstreet et al., 2005). Similar to many other gene targets, genetically induced permanent loss of AChE activity remains poorly studied due to the severe peripheral effects of AChE knockout, which result in early mortality (typically by four months of age) and pronounced motor abnormalities (Hrabovska et al., 2005). In contrast, AChE knockdown in the hippocampus has been shown to increase anxiety- and depression-like behaviors, as well as susceptibility to social stress — effects that can be reversed by SSRI fluoxetine treatment (Mineur et al., 2013).

The muscarinic hypothesis of depression, an extension of the cholinergic hypothesis, is supported by several additional findings. Many antidepressants, particularly TCAs such as amitriptyline or protriptyline, have a high affinity for antagonizing muscarinic receptors (Richelson, 1994). While these antimuscarinic properties are traditionally associated with adverse effects, such as dry mouth, constipation, blurry vision, cognitive impairment, and urinary retention (Remick, 1988), their potential role in the antidepressant effects should also be considered. For example, TCAs are often more effective than SSRIs and are considered a second-line treatment when SSRIs fail to produce a response (Anderson, 1998). The efficacy may, in part, stem from the cholinergic component of TCAs' mechanism of action. Moreover, no significant nicotinic receptor activity has been observed with imipramine, amitriptyline, or nortriptyline (Rathbun & Slater, 1963), suggesting that the cholinergic effects of TCAs are primarily related to muscarinic receptor blockade.

Similar to acetylcholinesterase inhibitors, direct enhancement of cholinergic tone via muscarinic receptors has been linked to depressive symptoms. Muscarinic agonists such as arecoline and oxotremorine have been shown to worsen mood in both healthy individuals and bipolar patients (K. Davis et al., 1987; Nurnberger et al., 1983). Furthermore, the behavioral effects of physostigmine were attenuated by the muscarinic receptor antagonist atropine (Janowsky et al., 1973; Janowsky et al., 1983), indicating that these effects are mediated through central muscarinic mechanisms.

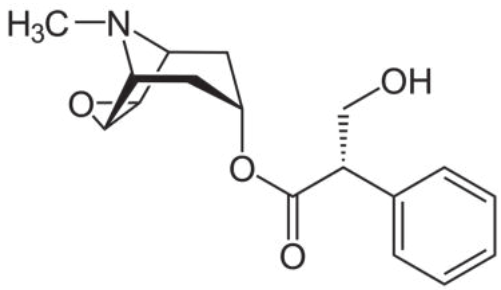


Figure 1: Molecular structure of the nonselective muscarinic receptor antagonist scopolamine, adapted from Wikipedia.

1.3.1 Scopolamine as a Novel Rapid-Acting Antidepressant

Scopolamine is an alkaloid drug found in certain nightshade plants (Solanaceae), which acts as a nonselective muscarinic receptor antagonist. Scopolamine butylbromide, a form that does not cross the blood-brain barrier, has been widely used for several years as an antispasmodic agent (Tytgat, 2007).

Although the cholinergic hypothesis of depression dates back to the 1970s, it did not receive serious attention until 2006, when clinical studies showed that scopolamine hydrobromide (HBr) — a form that crosses the blood-brain barrier — produces rapid antidepressant effects (Furey & Drevets, 2006). In these studies, currently depressed patients who met DSM-IV criteria for MDD or bipolar disorder underwent six randomized sessions, receiving either scopolamine (4 μg/kg) or placebo for three sessions each, in a crossover design. Significant reductions in depression (Montgomery-Asberg Depression Rating Scale, MADRS) and anxiety (Hamilton Anxiety Rating Scale, HARS) scores, as well as global improvement of patients' overall condition (Clinical Global Impressions-Improvement, CGI-I), were observed after the first dose of scopolamine and remained sustained during the placebo phase that followed. Unlike conventional antidepressants, which require weeks to produce a response, scopolamine appeared to exert antidepressant effects after just a single dose.

A few years later, Furey and Drevets expanded their initial study and found that while scopolamine produced rapid antidepressant effects in both men and women, the magnitude of response was significantly greater in women (Furey et al., 2010). Furthermore, scopolamine has been shown to be effective in individuals with treatment-resistant depression (Ellis et al., 2014). However, not all studies replicated these findings. Notably,

a similar methodology was employed, but no significant reductions in depressive symptoms or correlations with brain-derived neurotrophic factor (BDNF) plasma levels were observed (Park et al., 2019). The authors suggested that the higher treatment resistance (average past number of medication trials was greater than three), fewer treatment-naïve individuals, a greater proportion of inpatients, and higher baseline MADRS scores in their sample may explain the lack of significant effects compared to earlier studies.

Our systematic review of randomized controlled trials (RCTs) indicates that scopolamine exerts antidepressant effects of varying intensity (Moćko et al., 2023). The effects of intramuscular and intravenous administration are inconsistent, whereas oral scopolamine, when used as an adjunct to the SSRI citalopram, may be associated with beneficial clinical outcomes; however, this finding is reported in only one study. Nevertheless, it offers hope that scopolamine could be effective when administered orally in combination with other treatments, although further research is needed.

Numerous preclinical studies have confirmed the antidepressant properties of scopolamine. Antidepressant-like effects of scopolamine have been observed in both naïve and stressed animals across various behavioral parameters, including decreased immobility in the TST and FST, as well as decreased latency to feed in the novelty suppressed feeding test (NSFT) (Katz & Hersh, 1981; Navarria et al., 2015; Pałucha-Poniewiera et al., 2017; Voleti et al., 2013; Witkin et al., 2014).

In a reserpine-induced model of depression, subchronic scopolamine treatment (25 μg/kg, IP, three consecutive days) significantly reduced the reserpine-induced increase in immobility time in the FST in male ICR mice (Yu et al., 2019). Furthermore, scopolamine reversed the reserpine-induced downregulation of the serotonin transporter (5-HTT), BDNF, and tryptophan hydroxylase 1 (TPH1) in the hippocampus and PFC.

Studies in male C57BL/6J mice have also shown that the pro-depressive effects of the acetylcholinesterase inhibitor physostigmine in the TST were reversed by acute scopolamine treatment (0.5 mg/kg, IP) (Mineur et al., 2013).

Despite its promising potential as a rapid-acting and robust antidepressant, scopolamine also induces considerable adverse effects, including memory impairment, drowsiness, and visual disturbances (Renner et al., 2005), which hamper its use as a psychiatric drug. The therapeutic option that could help reduce the side effects of scopolamine would be its coadministration at lower doses with other substances with similar antidepressant

properties.

1.4 Glutamatergic System in Depression

Given the significant limitations of current pharmacotherapeutic treatments for depression that are based on the monoamine hypothesis, glutamatergic-related mechanisms also hold promise for developing more effective therapeutic interventions. Glutamate is the principal neurotransmitter in the brain, which is responsible for the bulk of excitatory synaptic transmission, regulating a multitude of processes including cognitive and motor functions, pain perception, and mood (Sanacora et al., 2012; Wozniak et al., 2012).

Emerging evidence from clinical, postmortem, and preclinical research strongly implicates dysregulation of glutamatergic transmission in MDD. Depressive symptoms are linked to structural abnormalities, network, and connectivity impairments in the cortical and limbic regions of the brain, which are associated with dysfunctions in excitatory glutamate neurons and inhibitory GABA interneurons. Metaanalysis of studies employing proton magnetic resonance spectroscopy has revealed reduced cortical levels of glutamine and glutamate in patients with depression (Moriguchi et al., 2019). Moreover, histological studies have shown glial cell reduction in the prefrontal cortex of depressed subjects (Öngür et al., 1998).

Preclinical studies using stress-based models of depression have shown a prominent impact of stress on glutamate release. Specifically, acute exposure to stress rapidly increases glutamate transmission in several cortical and limbic areas, including the PFC, hippocampus, and amygdala, which morphological alterations are visible in patients with MDD (Bagley & Moghaddam, 1997; Reznikov et al., 2007), where it positively effects the enhancement of excitatory synapses, synaptic plasticity, and working memory (Musazzi et al., 2015). Interestingly, such acute activation of glutamate neurotransmission has been also shown to be associated with upregulation of brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Zafra et al., 1991) — neurotrophins which levels are decreased in hippocampus and PFC of depressed subjects (Duman & Monteggia, 2006) and increased by chronic antidepressants' treatment (Nibuya et al., 1995).

Conversely, chronic stress and depression seem to be associated with a sustained increase in extracellular glutamate in PFC (S.-X. Li et al., 2018). Additionally, glial cells,

which play a critical role in regulating glutamate neurotransmission, were also found to be deficient following chronic stress (Sanacora & Banasr, 2013). Physiologically, since there are no enzymes able to metabolize glutamate in the extracellular space, excessive glutamate is primarily removed by astrocytes. As chronic stress continues to act, the ever-increasing glutamate level reaches excitotoxic levels, which can cause atrophy of dendrites, loss of synapses, reduction of synaptic transmission, and neuronal death (Choi, 1988; Musazzi et al., 2015). At that point, overall reduced glutamate neurotransmission as a result of a reduction in synaptic connectivity can be observed (Abdallah et al., 2018). Therefore, it seems relevant for novel antidepressants to rather activate than inhibit glutamate neurotransmission (Wierońska & Pilc, 2019).

Several clinical studies have shown that subanesthetic doses of a noncompetitive NMDA antagonist, ketamine, can induce rapid relief of depressive symptoms, which lasts for up to a week in patients affected by MDD (Berman et al., 2000; Zarate et al., 2006). In 2019, esketamine (Spravato), the S-enantiomer of ketamine, was approved by the FDA for the treatment of treatment-resistant depression.

1.4.1 Negative Modulation of Group II Glutamatergic Receptors

In addition to ionotropic receptors, metabotropic glutamate receptors (mGluRs) have also been proposed as attractive targets for novel therapeutic approaches against depression. Glutamate acts through three groups of G protein-coupled metabotropic glutamate receptors (mGluRs), which modulate synaptic function more slowly than ionotropic receptors. Group I consists of mGlu1 and mGlu5, group II includes mGlu2 and mGlu3, and group III comprises mGlu4, mGlu6, mGlu7, and mGlu8. Group I receptors are coupled to Gq/G11 proteins, leading to the activation of phospholipase Cβ, while groups II and III are primarily coupled to Gi/o proteins, resulting in the inhibition of adenylyl cyclase. Group I mGluRs are predominantly located postsynaptically, where their activation induces cell depolarization and enhances neuronal excitability. Conversely, group II and III mGluRs are generally found on presynaptic terminals, where they act as autoreceptors and suppress neurotransmitter release (Niswender & Conn, 2010). Nevertheless, group II mGluRs can be localized postsynaptically, where they can induce hyperpolarization (Muly et al., 2007), and long-term depression (LTD) (Bellone et al., 2008).

Postmortem studies have shown the elevated mGlu2/3 receptor levels in the PFC in

individuals with MDD (Feyissa et al., 2010). On the other hand, numerous preclinical studies suggest that mGlu2/3 antagonists and negative allosteric modulators, such as MGS0039, LY341495, TP0178894, VU6001966, and VU0650786, may produce rapid antidepressant-like effects (Chaki et al., 2004; Dong et al., 2022; Joffe et al., 2020; Pałucha-Poniewiera, Wierońska, et al., 2010; Podkowa, Pochwat, et al., 2016), suggesting that these receptors may be a promising target for novel antidepressants.

A highly conserved sequence within the same group of mGlu receptors makes it difficult to develop selective orthosteric ligands that can be used to differentiate the function of one receptor from the other (Conn & Pin, 1997). Through allosteric modulation, a better selectivity of a given compound for a specific receptor can be achieved, which in itself reduces the risk of side effects. Additionally, allosteric modulation has a more subtle effect on receptor activity compared to orthosteric ligands, allowing for the modulation of receptor activity. Therefore, the mGlu2 negative allosteric modulator (NAM), VU6001966, and mGlu3 NAM, ML289, were initially selected for combination with scopolamine.

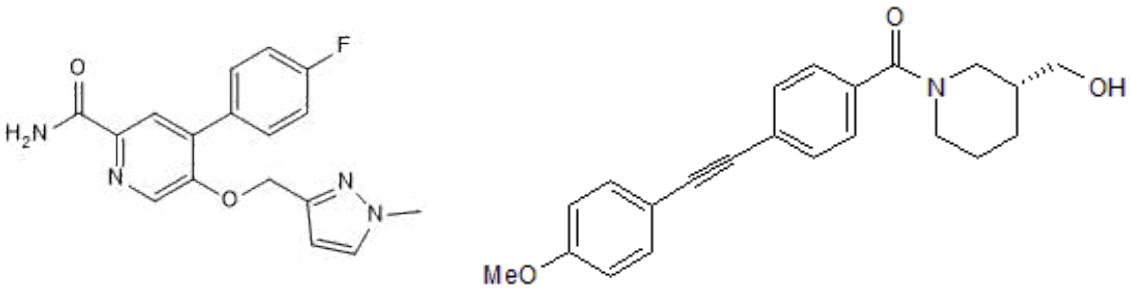


Figure 2: Molecular structures of (A) the mGlu2 negative allosteric modulator VU6001966 and (B) the mGlu3 negative allosteric modulator ML289, obtained from the Tocris Bioscience website.

Chapter 2

Research Objectives

In recent decades, there has been growing interest in repurposing existing drugs for new applications, and one such drug is scopolamine. The rapid and prolonged antidepressant effects of scopolamine, a nonselective antagonist of muscarinic cholinergic receptors, were first described in 2006. However, its use as a psychiatric drug has been limited due to significant adverse effects. This thesis aims to investigate whether the combined administration of scopolamine and the negative allosteric modulator of the mGlu2 receptor VU6001966 can enhance its antidepressant efficacy while reducing adverse effects.

This objective covers two key areas of research:

- Animal studies conducted on mice: to assess the antidepressant-like effects of the tested combination, its mechanism of action, and the risk of potential adverse effects.
- Animal studies conducted on rats: to investigate the effects of the tested compounds on cortical neurotransmission and behavior in rats.

Chapter 3

Materials and Methods

3.1 Animals and Housing

Male C57BL/6J mice (aged 6–7 weeks and weighing 23–25 g) and male Sprague-Dawley rats (weighing 250–350 g), obtained from Charles River Laboratories (Germany), were housed individually in cages with food and tap water provided ad libitum. The animals were kept under standard laboratory conditions of lighting (12-h light/dark cycle), humidity (55 ± 10%), and temperature (22 ± 2°C). Each experimental group comprised six to ten animals, and no animal was used more than once. Behavioral experiments were performed in a dedicated testing room during the light period (between 8 a.m. and 3 p.m.) of the light/dark cycle. All experiments were performed in accordance with the guidelines of the National Institutes of Health Animal Care and Use Committee and were approved by the Second Local Ethics Committee in Krakow, Poland. All efforts were made to minimize the number of animals used and their suffering. Considering that behavioral diversity is partially sex-dependent, and that the primary objective of the experiment was not to compare male and female behaviors, the study focused exclusively on male animals.

3.2 Compounds

Scopolamine hydrobromide and NBQX disodium salt (Tocris Cookson Ltd., Bristol, UK) were dissolved in 0.9% NaCl, while VU6001966, ML289, and ANA-12 (Tocris Cookson Ltd., Bristol, UK) were prepared in 0.5% methylcellulose with the addition of 2% DMSO. Groups of control animals were randomly chosen and received equal volumes of

0.9% NaCl. The tested compounds were prepared immediately before administration and administered intraperitoneally (IP) at a constant volume of 10 ml/kg for mice and 2 ml/kg for rats. The doses and the treatment schedules were selected based on the literature and previous studies (Cieślik et al., 2023; Joffe et al., 2020; Pałucha-Poniewiera et al., 2019; Podkowa, Podkowa, et al., 2016). Ketamine and xylazine hydrochloride, used for rat anesthesia, were obtained from Biowet Pulawy (Pulawy, Poland). All necessary chemicals of the highest purity used for analysis by high-performance liquid chromatography (HPLC) were obtained from Merck (Warsaw, Poland). O-phthalaldehyde (OPA) from Sigma-Aldrich (Poznan, Poland) was used to derivatize glutamate to an electroactive compound.

3.3 Studies Conducted on Mice

3.3.1 Unpredictable Chronic Mild Stress Procedure (UCMS)

UCMS model of depression was established in male C57BL/6J mice, following a previously outlined methodology (Rafało-Ulińska et al., 2022). The mice were randomly divided into two groups: unstressed (referred to as 'NS') and those that underwent the UCMS procedure (referred to as 'UCMS'). To eliminate additional stress from olfactory, visual, and auditory stimuli, these two groups were housed in separate rooms. After ten days of adaptation to the room conditions, the UCMS procedure was initiated, with stressors and their durations outlined in Table 1.

Two stressors were chosen daily, with the whole stress procedure lasting 14 days. Importantly, consecutive days did not involve the same stressor to ensure unpredictability, with a minimum gap of two hours between stressors. On the 15th day after the procedure initiation, the animals were treated with a vehicle or tested compounds, administered either once or subchronically (for four consecutive days). In experiments investigating the role of AMPA and TrkB receptor inhibitors in antidepressant-like response of the tested drug combination, NBQX or ANA-12 were injected 10 and 30 minutes, respectively, before the mixture of scopolamine and VU6001966. Then, 24 hours after the last treatment, behavioral tests were performed. Control mice underwent the same treatment and testing regimen as the UCMS mice. At the end of the study, the animals were sacrificed by decapitation, and specific brain structures, namely the PFC and hippocampus, were

Stressor	Duration
restraint stress	1 h
cage tilting 45°	6 h
wet beddind	2 h
predator smell (rat)	2 h
removal of sawdust	1 h
placing a mouse in the cage of another mouse	2 h
two male mice in one cage	2 h
$3-6$ individuals in a cage with 37°C water	30 min
overcrowding (12 individuals)	1 h

Table 1: Stressors used in the unpredictable chronic mild stress protocol.

collected and frozen at a temperature of -80°C.

3.3.2 Behavioral Tests for Assessing Antidepressant-Like Effects

After a 24-hour interval following the last treatment, three parameters reflecting the core symptoms of depression were analyzed. These parameters included reduced grooming time in the splash test, signifying apathy; diminished sucrose preference, indicative of anhedonia; and decreased periods of immobility observed in both the TST and FST tests, resembling behavioral despair. However, in accordance with Resolution No. 52/2022 of the National Ethics Committee for Animal Experiments dated December 16, 2022, the FST test has been removed from the list of performed tests in subsequent experiments.

Splash Test

The splash test was performed as previously described (Pałucha-Poniewiera et al., 2021) with slight modifications. The test was conducted under dimmed lighting in a room where the animal was acclimated for 30 minutes. To trigger self-grooming behavior, a high-viscosity 10% sucrose solution was sprayed onto the dorsal coat of the mice. The sprayer consistently delivered the sucrose solution (approximately 0.2 ml), with each mouse receiving five sprays. The duration of grooming behavior — including nose/face grooming, head washing, and body grooming — was recorded over a five-minute period.

Sucrose Preference Test (SPT)

The SPT was conducted following the established protocols (Pałucha-Poniewiera et al., 2021; Strekalova & Steinbusch, 2010), with minor modifications. One day before the test, mice were allowed to consume a palatable 2.5% sucrose solution for 2 hours to diminish the effects of neophobia. Then, the actual SPT started, where mice had unrestricted access to two bottles (1% sucrose solution or tap water) for 24 hours, with the positions of the bottles switched after 12 hours. No prior food or water deprivation was applied before the test. At the beginning and end of the test, the bottles were weighed, and liquid consumption was calculated. The sucrose preference rate was calculated using the following equation:

$$\text{Sucrose preference}\,[\%] = \frac{\text{Quantity of sucrose solution consumed}}{\text{Quantity of sucrose solution consumed} + \text{Quantity of water consumed}} \times 100\%$$

Tail Suspension Test (TST)

The TST was conducted according to a previously described method (Steru et al., 1985). In this procedure, each mouse was suspended by its tail approximately 75 cm above the floor, using adhesive tape placed about 1 cm from the tip of the tail. The total immobility duration was recorded for 6 min. The mice were considered immobile only when they either hung passively or remained completely motionless (Steru et al., 1985).

Forced Swim Test (FST)

The FST was carried out in accordance with the protocol previously used in our laboratory (Podkowa, Podkowa, et al., 2016). Mice were forced to swim individually in glass cylinders (height: 25 cm, diameter: 10 cm) filled with water (depth: 10 cm) at 23 °C. The animals remained in the cylinder for 6 min. The total duration of immobility was measured during the last 4 min of the test. Immobility was recognized when a mouse floated passively in an upright position with its head positioned above the water level (Porsolt et al., 1977).

3.3.3 Locomotor Activity Test for Assessing General Activity

The spontaneous locomotor activity was recorded individually for each animal according to the previously described procedure to eliminate the non-specific effects of tested

drugs (Pałucha-Poniewiera, Brański, et al., 2010). The measurement system consisted of plexiglas locomotor activity cages (24 × 24 × 20cm) in a photobeam activity system (Opto-Varimex 4, Columbus Instruments, USA), where the animals were individually placed. After each trial, the cages were thoroughly washed and dried. The total number of ambulations was recorded in 6-minute intervals for a total of 30 minutes. Each group consisted of 8–10 mice.

3.3.4 Behavioral Tests for Assessing Memory Function

For the estimation of cognition processes, the object location test (OLT) and the novel object recognition test (NORT) were used as previously described (Denninger et al., 2018). During the habituation session, each animal was exposed to the black plastic box ($30 \times 43 \times 22$cm) without any object for 6 minutes. After inter-trial interval lasting 20 minutes, habituation was repeated 2 times for a total of 3 habituation sessions. Twenty-four hours later, the animals were placed in the experimental box for 10 minutes, where two different objects were positioned longitudinally. Afterwards, the animals were returned to their home cages for 20 minutes.

Object Location Test (OLT)

Twenty minutes after the training trial, the actual OLT test was performed. The animals were returned to the experimental box for 10 minutes, where the position of one object was changed, resulting in the two objects being placed diagonally. During the OLT, the time each animal spent exploring each object was recorded. Afterwards, the animals were returned to their home cages for 20 minutes.

The exploration time of the moved object was calculated using the following equation:

$$\text{Moved object investigation time}\,[\%] = \frac{\text{Time with novel location}}{\text{Time with novel location} + \text{Time with familiar location}} \times 100\%$$

If spatial memory was intact, the animal spent more time exploring the object whose location within the arena had been changed.

Novel Object Recognition Test (NORT)

Twenty minutes after finishing the OLT, the NORT test was performed. The mice were returned to the box for 10 minutes, where the object that was not moved during

the OLT was changed to a novel object. Similarly to the OLT, the time spent by the animal exploring each object was measured. The exploration time of the novel object was calculated using the following equation:

$$\text{Novel object investigation time}\,[\%] = \frac{\text{Time with novel object}}{\text{Time with novel object} + \text{Time with familiar object}} \times 100\%$$

If non-spatial memory was intact, the animal spent more time exploring the novel object.

3.3.5 Western Blot Analysis

Tissue samples from the PFC and hippocampus were homogenized using ice-cold lysis buffer composed of 0.32 M sucrose, 20 mM HEPES (pH 7.4), 1× protease inhibitor cocktail, 5 mM NaF, 1 mM $NaVO_3$, and 1 mM EDTA. Homogenates were centrifuged at 2800 rpm for 10 min at 4°C. The obtained supernatant was then centrifuged at 12,000 rpm for 10 min at 4°C. Pellets obtained from this second centrifugation were then sonicated in a protein lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 1 mM $NaVO_3$, 5 mM NaF, and a protease inhibitor cocktail. The protein concentrations were quantified using a commercially available BCA kit (Thermo Scientific, USA).

Thirty micrograms of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis (10%) and then transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). These membranes were blocked for 1 h using a 1% blocking solution (BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) made by Roche, Switzerland). Following the blocking step, the membranes were incubated overnight at 4 °C with the primary antibodies. The primary antibodies used included anti-mTOR (mTOR 1:1000; Cell Signaling Technology, USA), anti-phospho-mTOR (pmTOR, S2481, 1:1000; Abcam, USA), anti-phospho-eEF2 (pheEF2 (phospho T56) 1:1000; Abcam, USA), anti-eEF2 (eEF2 1:1000; Abcam, USA), anti-PSD95 (PSD 1:1000; Abcam, USA), anti-phospho-TrkB (pTrkB (Tyr816) 1:1000; ABN1381, Sigma-Aldrich, Germany), anti-TrkB (TrkB 1:1000; Cell Signaling Technology, USA), anti-BDNF (BDNF 1:1000; ab108319, Abcam, USA). On the following day, the membranes were washed three times for 10 min in Tris-buffered saline with Tween (TBS-T) and incubated for 60 min with corresponding secondary IgG-peroxidase-conjugated antibodies: horse anti-mouse IgG (1:1000; Vector

Laboratories, USA), goat anti-rabbit IgG (1:1000; Vector Laboratories, USA), and rabbit anti-goat IgG (1:5000; Abcam, USA). After this incubation, the membranes were washed three times for 10 min with TBS-T. In the final step, the blots were incubated with a detection reagent (Bio-Rad, USA). The signal emanating from the tested proteins was captured and quantified using a Fuji Las 1000 system and Fuji Image Gauge V4.0 software. A primary monoclonal antibody, specifically glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:500; Millipore, Germany), was indicated on each blot to verify the transfer and loading accuracy. The final result is presented as the ratio of the optical density of a particular protein to the optical density of GAPDH. Each experiment was replicated twice for reliability.

3.4 Studies Conducted on Rats

3.4.1 Behavioral Tests for Assessing Antidepressant-Like Effects and General Activity

Forced Swim Test (FST)

The studies were carried out according to the method of Porsolt et al. (Porsolt et al., 1978). The rats were placed in glass cylinders (height 40 cm, diameter 20 cm) containing 15 cm of water, maintained at 24°C. Two swim sessions were conducted: an initial 15-minute pretest followed 24 hours later by a 5-minute test. Following both sessions, rats were removed from the cylinders and returned to their home cages. Behavioral scoring was performed according to Detke et al. (Detke et al., 1995) and during the 5 min test session three different behaviors were rated: 1) immobility — rat was judged to be immobile when it remained floating passively in the water; 2) swimming — rat was judged to be swimming if it was making active swimming motions, more than necessary to maintain its head above water solely; 3) climbing — rat was judged to be climbing when it was making active movements in and out of the water with its forepaws, usually directed against the walls.

3.4.2 Locomotor Activity Test

Spontaneous locomotor activity was recorded individually for each animal in Opto-Varimex (Columbus Instruments, Columbus, OH, USA) plexiglass chambers (43×43 cm), where the animals were individually placed for an acclimation period of 60 min. Then, they were administered an IP drug injection. Immediately after injection, each rat was returned to the chamber. The total distance traveled during a 120-minute experimental session was measured and stored every 5 minutes. Each cage was surrounded by a 15×15 array of photocell beams located 3 cm from the floor surface. Interruptions of the photobeams were interpreted as horizontal activity, and the distance traveled was converted into centimeters. All the data were analyzed using Auto-track software (Columbus Instruments, USA).

3.5 Brain Microdialysis Procedure

Rats were anesthetized with 75 mg/kg ketamine and 10 mg/kg xylazine intramuscularly and placed on a stereotaxis apparatus. Microdialysis probe (MAB 4.15.4Cu, AgnTho's AB, Sweden) was lowered slowly into the FCX at the following stereotaxis coordinates (mm): AP +2.7, L +0.8, and V -6.5 from the bregma and dura surface according to the atlas (Paxinos & Watson, 1998). The active surface of the dialysis probe was 4 mm. Seven days after implantation, probe inlets were connected to a syringe pump (BAS, West Lafayette, IN, USA), which delivered artificial cerebrospinal fluid (aCSF) composed of 147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$ and 1.0 mM $MgCl_2$ at a constant flow rate of 2 μl/min. The monitoring of extracellular levels of neurotransmitters has been performed in freely moving animals. Five baseline samples were collected every 20 minutes after the washout period of 2 hours. The respective drugs were administered, and dialysate fractions were collected for the next 120 minutes. As the experiment ended, the rats were terminated, and their brains underwent histological examination to validate probe placement.

3.6 Extracellular Concentration of DA, 5-HT, Glutamate, and GABA

Extracellular DA and 5-HT levels were analyzed using a UHPLC Ultimate 3000 system (Thermo Fisher Scientific, Sunnyvale, CA, USA), an electrochemical detector ECD-3000 RS with a 6020RS-omni coulometric cell, 6011RS ultra coulometric analytical cell, and a Hypersil Gold C18 analytical column (3 μm, 100 $\times$ 3 mm; Thermo Fisher Scientific, Sunnyvale, CA, USA). The mobile phase consisted of 0.1 M KH_2PO_4 buffer at pH 3.8, 0.5 mM Na_2EDTA, 100 mg/l 1-octanesulfonic acid sodium salt, and 3.2% methanol. The flow rate during the analysis was set at 0.6 ml/min, and the applied potential of an omni coulometric cell was 600 mV, whereas the potential of an ultra-coulometric analytical cell was $E_1 = -50\,\text{mV}$ and $E_2 = 300\,\text{mV}$ with a sensitivity set at 10 nA/V. The chromatographic data were processed by the Chromeleon v.7.280 (Thermo Fisher Scientific, Sunnyvale, CA, USA) software package run on a personal computer. The detection limit for DA was 0.002 pg/10 μl and 0.01 pg/10 μl for 5-HT. Glutamate and GABA levels in the extracellular fluid were measured by HPLC with electrochemical detection after the derivatization of samples with OPA/sulfite reagent to form isoindole-sulfonate derivatives (Rowley et al., 1995). The data were processed using Chromax 2005 (Pol-Lab, Warszawa, Poland) software on a personal computer. The limit of detection of glutamate and GABA in dialysates was 0.03 ng/10 μl and 6.4 pg/10 μl, respectively.

3.7 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 10.3.1 (GraphPad Software, San Diego, CA, USA) and STATISTICA v.13.3 StatSoft Inc. 1984–2011 (TIBCO Software Inc., Palo Alto, CA, USA). The number of animals used varied across groups between 6 and 10. The distribution of variables and the homogeneity of variances were checked by the Shapiro–Wilk's test and Levene's test, respectively. Two-way ANOVA followed by Bonferroni's post hoc test was used to analyze the interactions between UCMS and tested drugs, AMPA/TrkB receptor blocking, cognitive effects, and Western blot results. The results from the remaining behavioral tests were assessed using a one-way ANOVA followed by Dunnett's post hoc test. Data illustrating locomotor activity were

evaluated by a two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test. Drug effects on DA, 5-HT, glutamate, and GABA release in the FCX were analyzed with two-way repeated measures ANOVA on normalized responses followed by Tukey's post hoc test (time course) and a two-way ANOVA followed by Bonferroni's post hoc test (total effect). All data are presented as mean ± SEM. P values lower than 0.05 were regarded as statistically significant.

Chapter 4

Results

4.1 Studies Conducted on Mice

4.1.1 Behavioral Effects of a Single Administration of Scopolamine

In the splash test, two-way ANOVA revealed no interaction between UCMS and scopolamine treatment [$F(2, 50) = 5.288$, $P = 0.0083$; Fig. **3B**]. There was a significant main effect of the UCMS procedure [$F(1, 51) = 61.06$, $P < 0.0001$], but no main effect of scopolamine treatment [$F(2, 51) = 0.02423$, $P = 0.9761$]. Post hoc Bonferroni's multiple comparisons test showed a significant decrease in grooming in vehicle-treated UCMS mice compared with NS controls ($P < 0.0001$). Similar results were obtained in the sucrose preference test (SPT), where two-way ANOVA revealed no interaction between UCMS and scopolamine treatment [$F(2, 48) = 0.2875$, $P = 0.7514$; Fig. **3C**]. There was a significant main effect of the UCMS procedure [$F(1, 48) = 70.13$, $P < 0.0001$], but no main effect of scopolamine treatment [$F(2, 48) = 1.129$, $P = 0.3318$]. Post hoc Bonferroni's multiple comparisons test showed a significant decrease in sucrose preference in vehicle-treated UCMS mice compared with NS controls ($P < 0.0001$). In the tail suspension test (TST), two-way ANOVA revealed no interaction between UCMS and scopolamine [$F(2, 47) = 3.054$, $P = 0.0566$; Fig. **3D**], and neither a main effect of the UCMS procedure [$F(1, 47) = 1.071$, $P = 0.3060$] nor of scopolamine treatment [$F(2, 47) = 0.027$, $P = 0.973$]. In the forced swim test (FST), two-way ANOVA revealed no interaction between UCMS and scopolamine [$F(2, 46) = 3.170$, $P = 0.0513$; Fig. **3E**].

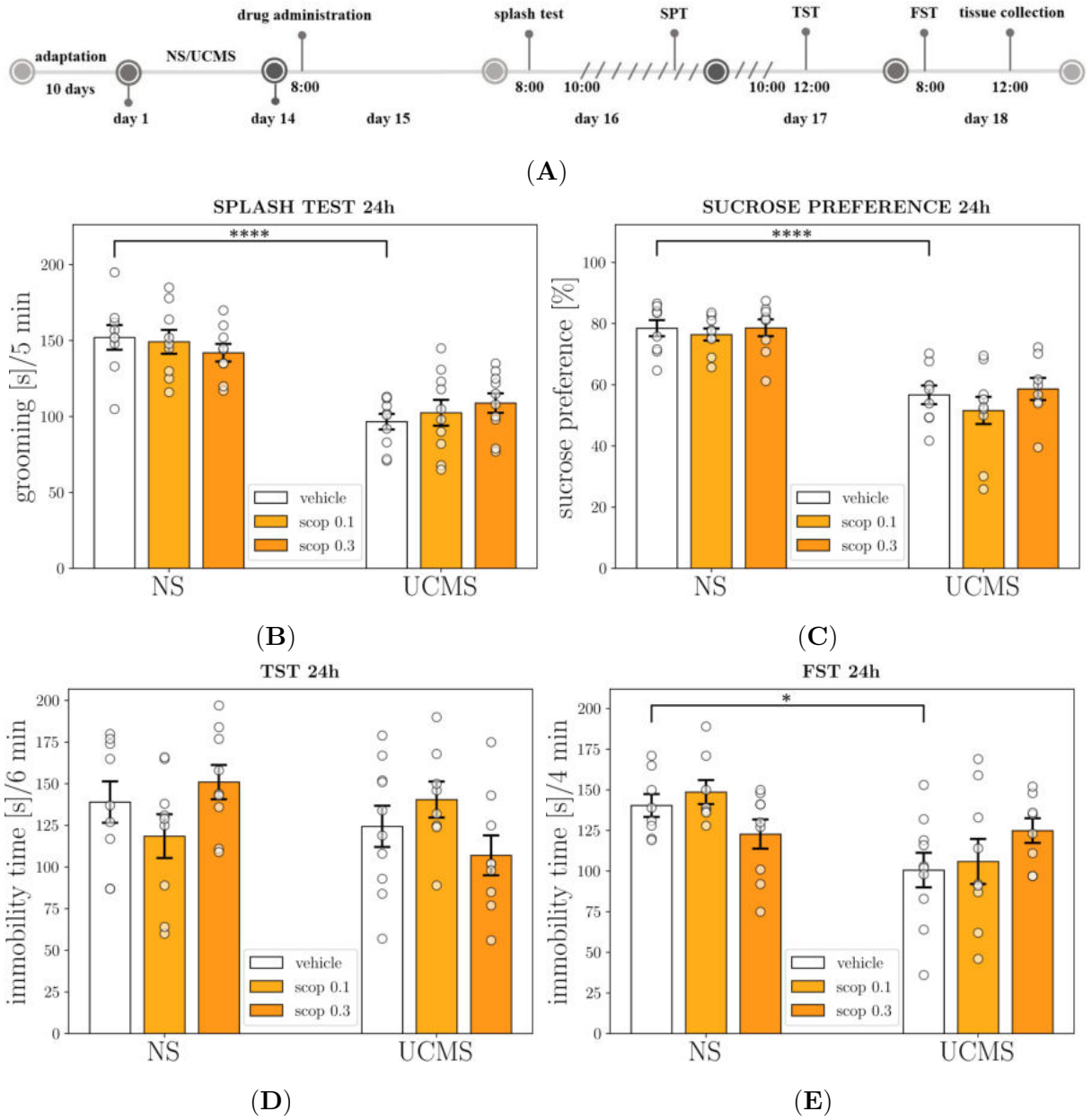


Figure 3: The antidepressant-like effects of a single administration of scopolamine in the UCMS model of depression. (A) Schematic representation of the experimental schedule. After a 10-day adaptation period to room conditions, the UCMS procedure was initiated and continued for 14 days. On day 15, the animals received a single administration of either vehicle or scopolamine. Twenty-four hours later, the splash test was performed, followed two hours later by the SPT, which lasted for 24 hours. Two hours after completion of the SPT, the TST was conducted. On the following day, the FST was carried out, and four hours after its completion, the animals were sacrificed by decapitation for the collection of brain structures. Behavioral effects in (B) splash test, (C) SPT, (D) TST, and (E) FST. The values are expressed as the means ± SEM ($N = 8-10$) and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. $*P < 0.05$; $****P < 0.0001$ vs. the respective NS vehicle. NS — non-stressed; UCMS — unpredictable chronic mild stress.

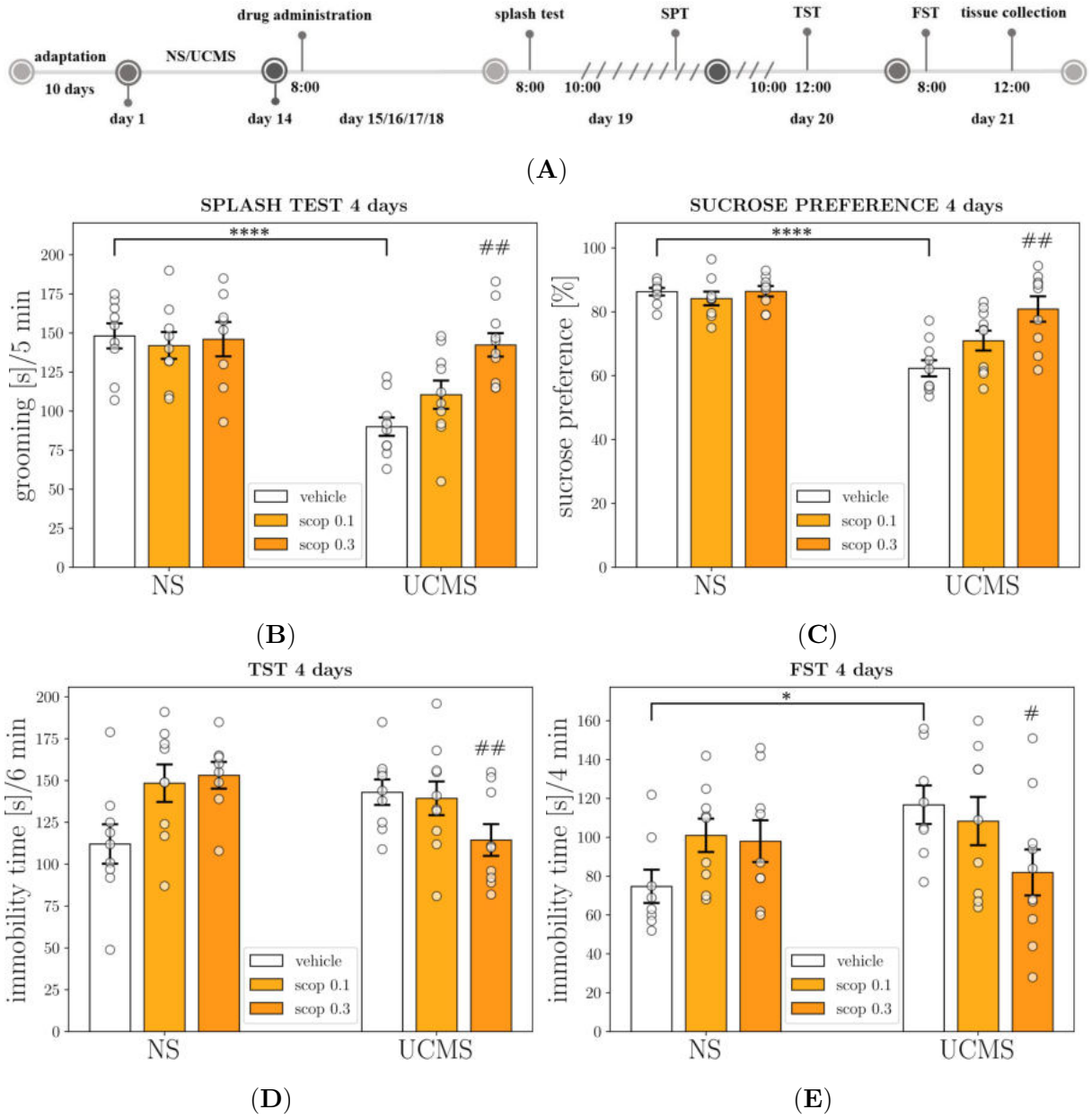


Figure 4: The antidepressant-like effects of four-day administration of scopolamine in the UCMS model of depression. (A) Schematic representation of the experimental schedule. After a 10-day adaptation period to room conditions, the UCMS procedure was initiated and continued for 14 days. Beginning on day 15, the animals received subchronic treatment with either vehicle or scopolamine. Twenty-four hours after the last administration, the splash test was performed, followed two hours later by the SPT, which lasted for 24 hours. Two hours after completion of the SPT, the TST was conducted. On the following day, the FST was carried out, and four hours after its completion, the animals were sacrificed by decapitation for the collection of brain structures. Behavioral effects in (B) splash test, (C) SPT, (D) TST, and (E) FST. The values are expressed as the means ± SEM ($N = 8 - 10$) and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. $*P < 0.05$; $****P < 0.0001$ vs. the respective NS vehicle; $\#P < 0.05$; $\#\#P < 0.01$ interaction: UCMS × scop. NS — non-stressed; UCMS — unpredictable chronic mild stress.

There was a significant main effect of the UCMS procedure [$F(1, 46) = 10.97$, $P = 0.0018$], but no main effect of scopolamine treatment [$F(2, 46) = 0.2351$, $P = 0.7914$]. Post hoc Bonferroni's multiple comparisons test showed a significant decrease in immobility in vehicle-treated UCMS mice compared with NS controls ($P = 0.0179$). These results indicate that a single administration of scopolamine was insufficient to reverse UCMS-induced depressive-like symptoms in mice.

4.1.2 Behavioral Effects of Four-Day Administration of Scopolamine

The effects of a four-day scopolamine treatment (0.1 and 0.3 mg/kg) on UCMS-induced behavioral alterations were examined. In the splash test, two-way ANOVA revealed significant interactions between UCMS and scopolamine [$F(2, 50) = 5.288$, $P = 0.0083$; Fig. **4B**], as well as main effects of UCMS [$F(1, 50) = 20.84$, $P < 0.0001$], and scopolamine treatment [$F(2, 50) = 4.741$, $P = 0.0130$], indicating reversal of the UCMS-induced apathy-like state. Bonferroni post-hoc test showed a significant reduction in grooming time in UCMS mice compared to non-stressed controls ($P < 0.0001$). In the SPT, similar findings were observed. Two-way ANOVA indicated a significant interaction between UCMS and scopolamine [$F(2, 50) = 6.236$, $P = 0.0038$; Fig. **4C**], along with main effects of UCMS [$F(1, 50) = 44.50$, $P < 0.0001$], and scopolamine [$F(2, 50) = 6.497$, $P = 0.0031$], suggesting reversal of UCMS-induced anhedonia-like behavior. Bonferroni post-hoc test confirmed a significantly reduced sucrose preference in stressed mice ($P < 0.0001$). In the TST, two-way ANOVA revealed a significant interaction between UCMS and scopolamine [$F(2, 48) = 6.048$, $P = 0.0045$; Fig. **4D**], but no main effects of stress [$F(1, 48) = 0.4797$, $P = 0.4919$] or scopolamine [$F(2, 48) = 1.431$, $P = 0.2490$] were observed. Bonferroni post-hoc tests showed no significant changes in immobility time between stressed and control mice ($P = 0.0967$). In the FST, two-way ANOVA also revealed a significant interaction between UCMS and scopolamine [$F(2, 47) = 3.683$, $P = 0.0327$; Fig. **4E**], and the Bonferroni's post-hoc test revealed a significantly increased immobility time of stressed mice compared to that of the unstressed controls ($P = 0.0320$). Although no effect of UCMS [$F(1, 47) = 1.620$, $P = 0.2094$], or scopolamine [$F(2, 47) = 1.012$, $P = 0.3712$] was observed. These results show that subchronic scopolamine treatment significantly alleviated stress-induced depressive-like behaviors in mice.

4.1.3 Effects of Scopolamine Four-Day Administration on eEF2, TrkB, mTOR, and PSD95 Proteins in the PFC

In the prefrontal cortex, a two-way ANOVA revealed an interaction between UCMS and scopolamine at a dose of 0.3 mg/kg [$F(1, 22) = 20.13$, $P = 0.0002$; Fig. **5A**], indicating a reversal of the UCMS-induced changes in phospho-eEF2/eEF2 ratio. Bonferroni post-hoc test indicated a significantly increased phospho-eEF2/eEF2 ratio in stressed mice compared to the NS controls ($P = 0.0004$; Fig. **5A**). Statistical analyses of Western blots in the PFC did not reveal any interaction between UCMS and scopolamine regarding the level of TrkB, mTOR, and PSD95 proteins ([$F(1, 29) = 1.974$, $P = 0.1706$]; [$F(1, 23) = 2.158$, $P = 0.1554$], and [$F(1, 27) = 0.4304$, $P = 0.5173$] for Fig. **5B–5D**, respectively). However, a clear trend to decrease the TrkB level between the vehicle group and UCMS group was observed ($P = 0.0894$, for Fig. **5B**). These results suggest that subchronic scopolamine treatment significantly reversed the stress-induced increase in the peEF2/eEF2 ratio in the PFC.

4.1.4 Effects of Scopolamine Four-Day Administration on eEF2, TrkB, mTOR, and PSD95 Proteins in the Hippocampus

In the hippocampus, two-way ANOVA did not show any interaction between UCMS and scopolamine ([$F(1, 24) = 1.190$, $P = 0.2861$]; [$F(1, 28) = 1.086$, $P = 0.3062$]; [$F(1, 24) = 0.03441$, $P = 0.8544$]; [$F(1, 29) = 0.008$, $P = 0.9283$] for Fig. **6A–6D**, respectively), suggesting that the levels of the tested proteins were unaffected by either stress or subchronic scopolamine treatment.

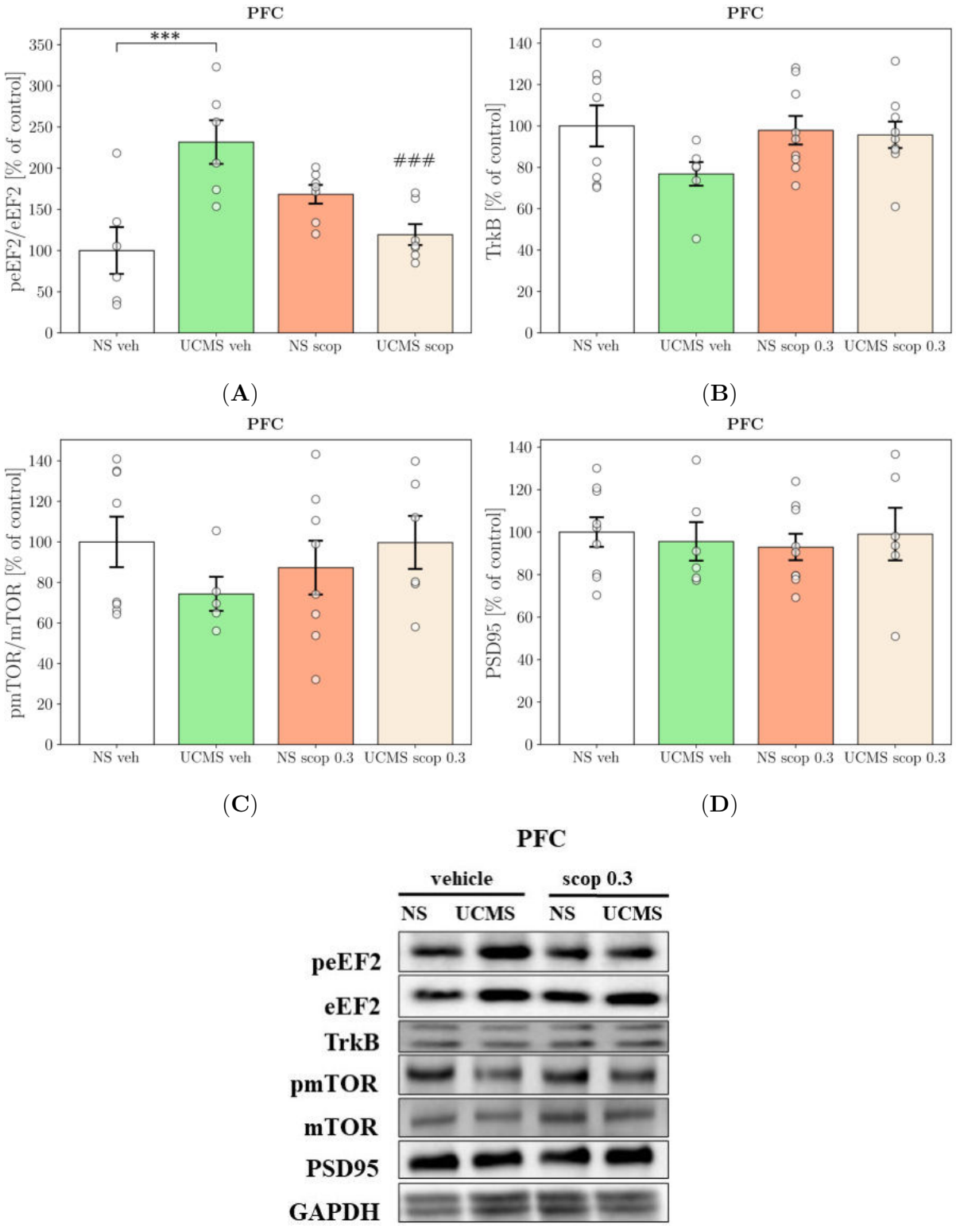


Figure 5: The effect of a four-day administration of scopolamine (0.3 mg/kg) on the (A) peEF2/eEF2, (B) TrkB, (C) pmTOR/mTOR, and (D) PSD95 protein levels analyzed by Western blot analysis in the PFC. The values are expressed as a percentage of changes vs. control levels ($N = 6 - 9$) and were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. $***P < 0.001$ vs. the respective NS vehicle; $\#\#\#P < 0.001$ interaction: UCMS × scop. NS — non-stressed; UCMS — unpredictable chronic mild stress.

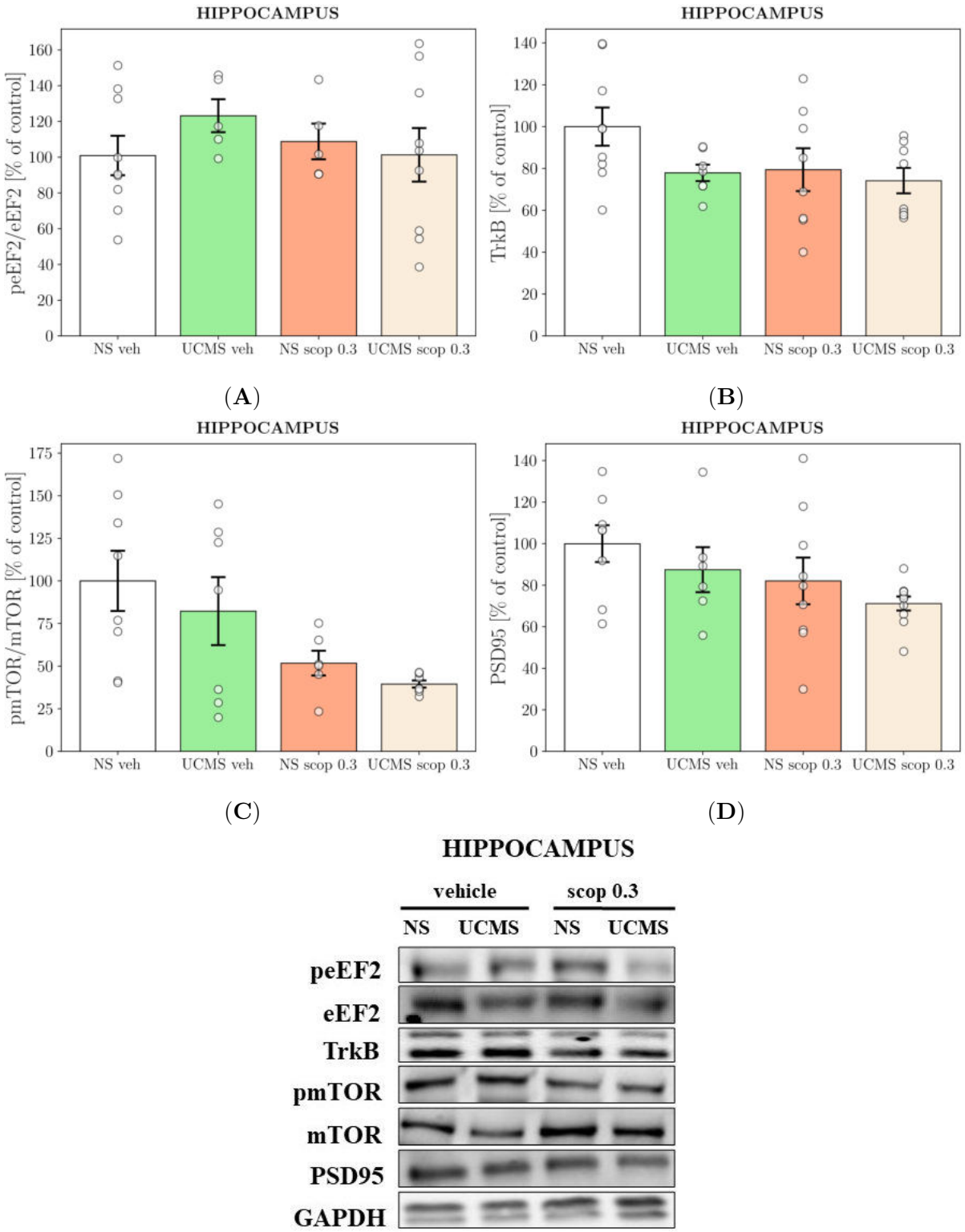


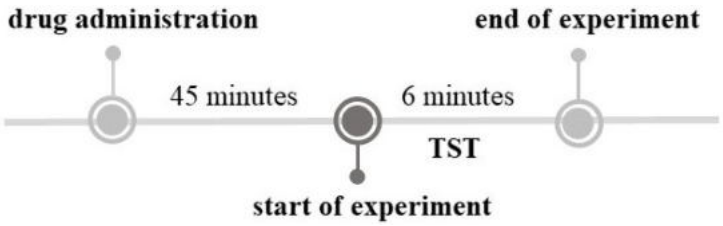
Figure 6: The effect of a four-day administration of scopolamine (0.3 mg/kg) on the (A) peEF2/eEF2, (B) TrkB, (C) pmTOR/mTOR, and (D) PSD95 protein levels analyzed by Western blot analysis in the hippocampus. The values are expressed as a percentage of changes vs. control levels ($N = 6 - 9$) and were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. NS — non-stressed; UCMS — unpredictable chronic mild stress.

4.1.5 Acute Antidepressant-Like Effects of mGlu2 NAM VU6001966 and mGlu3 NAM ML289

To investigate whether the selective mGlu2 NAM VU6001966 or mGlu3 NAM ML289 could modulate depressive-like behavior in the tail suspension test, mice were treated with varying doses of VU6001966 ($0.3 - 10$ mg/kg) and ML289 ($10 - 50$ mg/kg) 45 minutes prior to testing. One-way ANOVA revealed significant antidepressant-like effects of VU6001966 [$F(4, 37) = 8.016$, $P < 0.0001$; Fig. **7B**]. Dunnett's post hoc analysis revealed that VU6001966 at 10 mg/kg significantly reduced immobility time compared to the vehicle-treated control group ($P = 0.0009$). ML289 had no effect on immobility time [$F(3, 27) = 1.507$, $P = 0.2353$; Fig. **7C**]. Based on these results, VU6001966 was selected for combination with scopolamine in subsequent experiments.

4.1.6 Behavioral Effects of Four-Day Administration of VU6001966

One-way ANOVA showed the dose-dependent effects of VU6001966 ($1 - 10$ mg/kg, IP) on grooming time in the splash test [$F(3, 36) = 10.17$, $P < 0.0001$; Fig. **8B**]. Dunnett's post hoc tests revealed that VU6001966 significantly increased the grooming time (given at doses of 3 and 10 mg/kg, $P = 0.0423$ and $P < 0.0001$, respectively). The dose-dependent effects of VU6001966 on sucrose preference were also observed [$F(3, 33) = 12.55$, $P < 0.0001$; Fig. **8C**]. Dunnett's post hoc tests revealed that VU6001966 significantly increased the sucrose preference (given at a dose of 10 mg/kg, $P < 0.0001$). When applied at a dose of 1 mg/kg, VU6001966 did not affect any of the tested parameters. Subchronic VU6001966 treatment did not produce any effect on immobility time in the TST [$F(3, 31) = 2.012$, $P = 0.1326$; Fig. **8D**]. Taken together, these results show that subchronic VU6001966 treatment significantly alleviated stress-induced depressive-like behaviors in mice.



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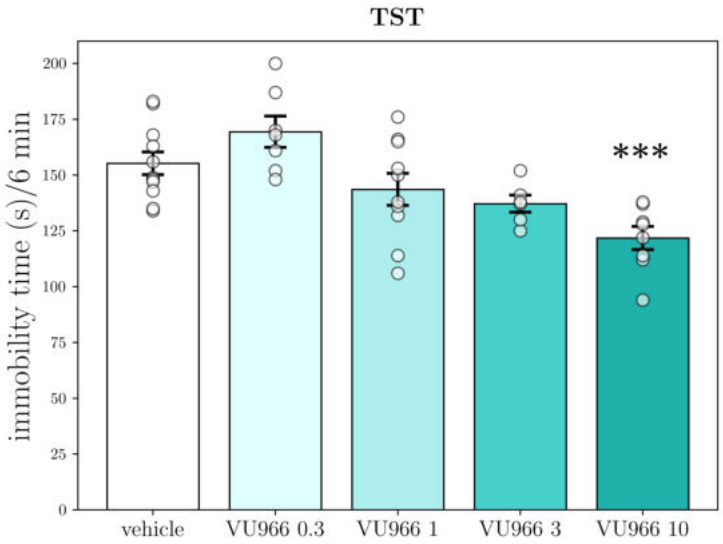

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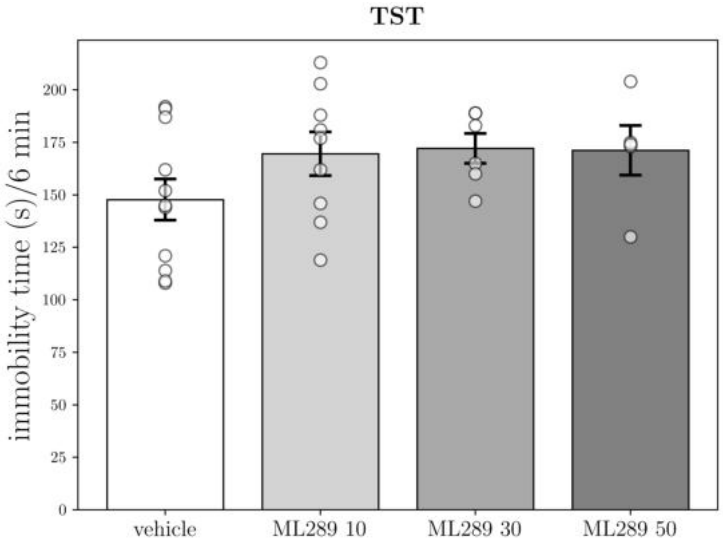

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Figure 7: Acute antidepressant-like effects of mGlu2 NAM VU6001966 and mGlu3 NAM ML289 in the tail suspension test (TST) in C57BL/6J mice. (A) Schematic representation of the experimental schedule. The TST was conducted 45 minutes after drug administration and lasted 6 minutes. Behavioral effects of (B) VU6001966 ($0.3-10$ mg/kg) and (C) ML289 ($10-50$ mg/kg). The values are expressed as the means ± SEM ($N = 5-10$) and were analyzed by one-way ANOVA followed by Dunnett's post hoc test. $***P < 0.001$ vs. vehicle.

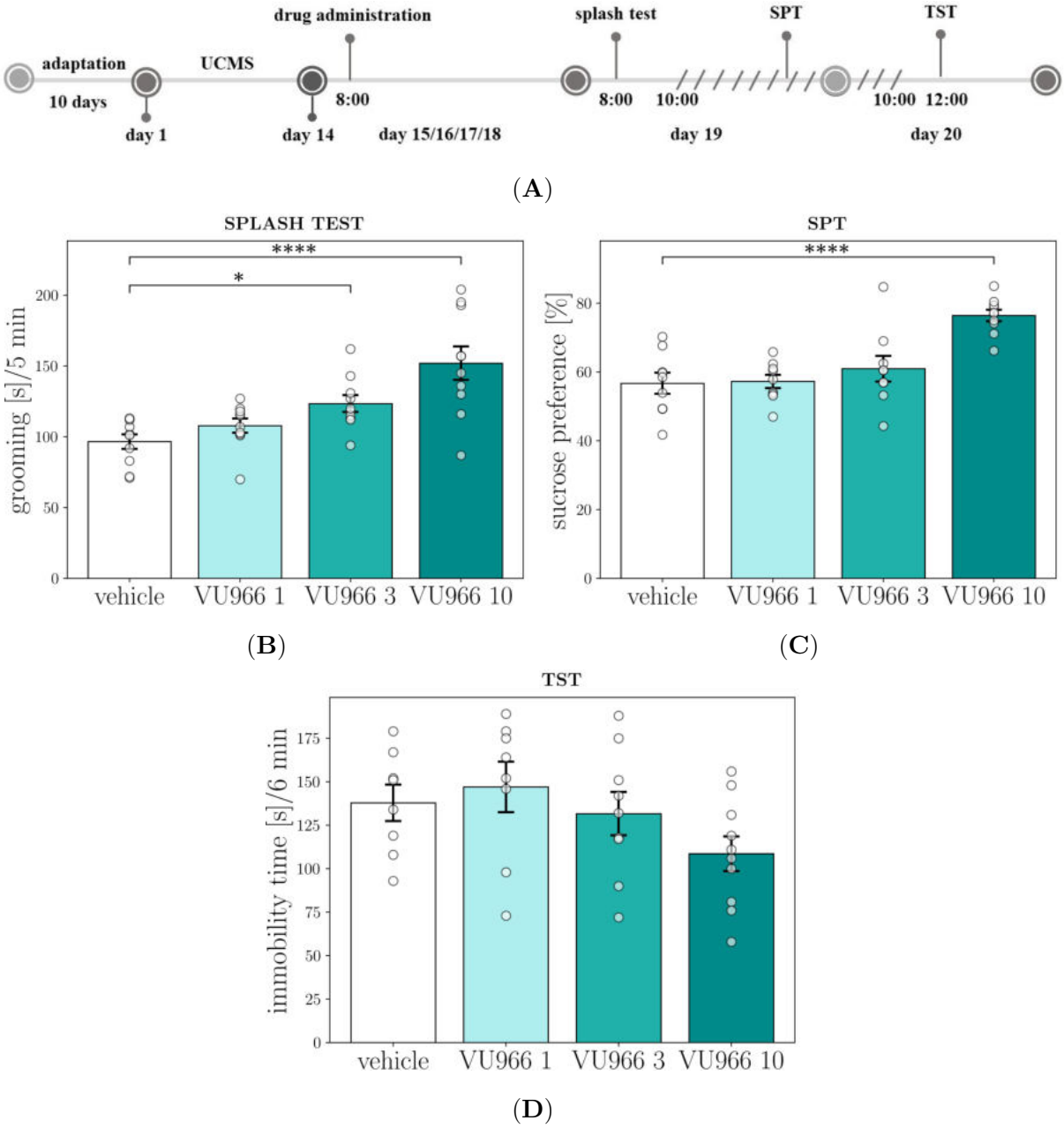


Figure 8: The antidepressant-like effects of four-day administration of VU6001966 in the UCMS model of depression. (A) Schematic representation of the experimental schedule. After a 10-day adaptation period to room conditions, the UCMS procedure was initiated and continued for 14 days. Beginning on day 15, the animals received subchronic treatment (four consecutive days) with either vehicle or VU6001966. Twenty-four hours after the last administration, the splash test was performed, followed two hours later by the SPT, which lasted for 24 hours. Two hours after completion of the SPT, the TST was conducted. Behavioral effects in the (B) splash test, (C) SPT, and (D) TST. Values are expressed as the mean ± SEM ($N = 8 - 10$) and were analyzed by one-way ANOVA followed by Dunnett's post hoc test ($*P < 0.05$; $****P < 0.0001$ vs. respective vehicle). UCMS — unpredictable chronic mild stress.

4.1.7 Behavioral Effects of Four-Day Coadministration of Scopolamine with VU6001966

To study the antidepressant-like effects of combined administration, the subeffective doses of 0.1 and 1 mg/kg for scopolamine and VU6001966, respectively, were selected for the experiment. In the splash test, a two-way ANOVA showed an interaction between UCMS and tested drugs [$F(3, 63) = 9.845$, $P < 0.0001$; Fig. **9B**], and effect of both UCMS procedure [$F(1, 63) = 5.558$, $P = 0.0215$], and drug treatment [$F(3, 63) = 15.51$, $P < 0.0001$], indicating a reversal of the UCMS-induced apathy-like state. Bonferroni's multiple comparisons test showed decreased grooming time in vehicle-treated UCMS group compared to the naïve mice ($P = 0.0044$), and showed that combined administration of scopolamine and VU6001966 reversed the UCMS-induced reduction in grooming ($P < 0.0001$) with no effect in NS mice ($P > 0.05$). In the SPT, statistical analysis also revealed a notable interaction between UCMS and tested drugs [$F(3, 60) = 5.893$, $P = 0.0014$; Fig. **9C**], as well as a main effect of the UCMS procedure [$F(1, 60) = 104.4$, $P < 0.0001$], but no main effect of the drugs [$F(3, 60) = 1.66$, $P = 0.1851$]. Bonferroni's multiple comparisons test showed decreased sucrose preference in vehicle-treated UCMS mice compared to NS controls ($P < 0.0001$). It was further demonstrated that in UCMS animals, an inactive dose of scopolamine given together with an inactive dose of VU6001966 significantly reduced stress-induced anhedonia ($P = 0.0044$), with no such effect in non-stressed animals ($P > 0.05$). In the TST, two-way ANOVA revealed no interaction between UCMS and the drugs [$F(3, 58) = 1.269$, $P = 0.2936$; Fig. **9D**] and no main effect of the drugs [$F(3, 58) = 1.77$, $P = 0.163$], but a significant main effect of the UCMS procedure was observed [$F(1, 58) = 28.57$, $P < 0.0001$]. No significant differences between vehicle-treated UCMS mice and NS controls were observed ($P > 0.05$), as well as between vehicle-treated naïve/UCMS mice and mice treated with a combination of scopolamine and VU6001966 ($P > 0.05$). Taken together, these results indicate that VU6001966 significantly enhances the antidepressant-like effects of subeffective doses of scopolamine in the UCMS model of depression in mice.

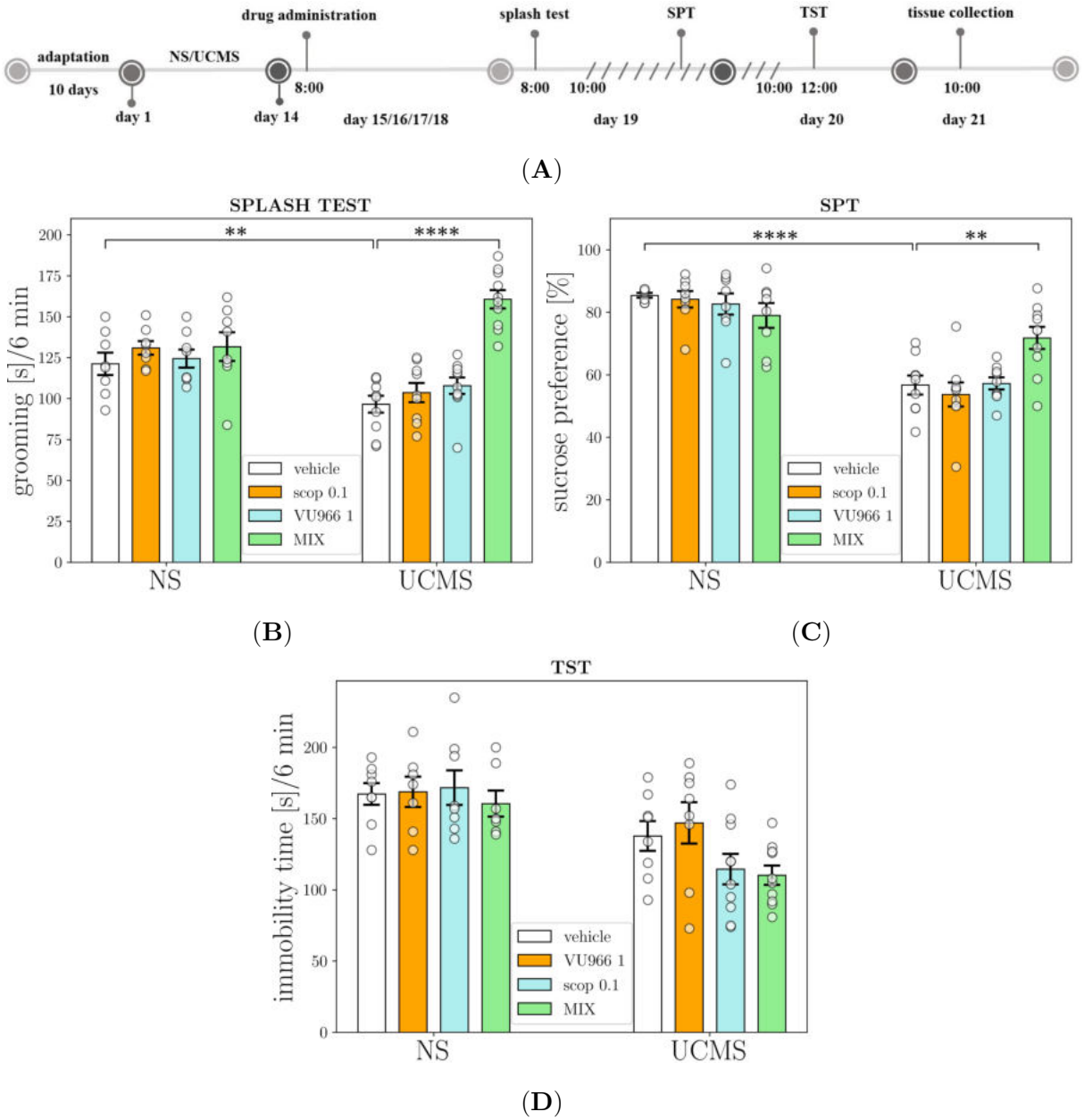


Figure 9: The antidepressant-like effects of four-day coadministration of scopolamine with VU6001966 in the UCMS model of depression. (A) Schematic representation of the experimental schedule. After a 10-day adaptation period to room conditions, the UCMS procedure was initiated and continued for 14 days. Beginning on day 15, the animals received subchronic treatment (four consecutive days) with either vehicle or the tested drugs. Twenty-four hours after the last administration, the splash test was performed, followed two hours later by the SPT, which lasted for 24 hours. Two hours after completion of the SPT, the TST was conducted. On the following day, the animals were sacrificed by decapitation for the collection of brain structures. Behavioral effects in (B) splash test, (C) SPT, and (D) TST. Values are expressed as the mean ± SEM ($N = 8 - 10$) and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test ($** P < 0.01$; $**** P < 0.0001$ vs. respective vehicle). NS — non-stressed; UCMS — unpredictable chronic mild stress; MIX — scopolamine 0.1 mg/kg + VU6001966 1 mg/kg.

4.1.8 Effects of the AMPA Receptor Antagonist NBQX on Scopolamine and VU6001966 Antidepressant Action

In the splash test, a two-way ANOVA revealed no interaction between NBQX (10 mg/kg) and tested combination [$F(1,31) = 2.299$, $P = 0.1396$; Fig. **10B**], and no main effect of the NBQX [$F(1,31) = 0.3559$, $P = 0.5551$], but a significant main effect of the tested combination was observed [$F(1,31) = 18.02$, $P = 0.0002$]. Bonferroni's post hoc tests confirmed increased grooming time in group treated with given mixture ($P = 0.0019$) compared to the UCMS vehicle group. A significant interaction between NBQX and a mixture of scopolamine and VU6001966 was observed also in the SPT [$F(1,35) = 10.24$, $P = 0.0029$; Fig. **10C**], and main effect of both tested combination [$F(1,35) = 7.66$, $P = 0.009$] and NBQX [$F(1,35) = 6.785$, $P = 0.0134$] were observed. However, this was mainly due to a significant decrease in sucrose consumption in NBQX-treated mice compared to the UCMS vehicle group ($P = 0.0006$). In the TST, a significant interaction between NBQX and the combination of scopolamine and VU6001966 was observed [$F(1,34) = 10.43$, $P = 0.0027$; Fig. **10D**], without significant main effects of either the tested combination [$F(1,34) = 0.7786$, $P = 0.3838$] or NBQX [$F(1,34) = 0.138$, $P = 0.7124$]. Bonferroni's post hoc tests confirmed decreased immobility in mice treated with the combination ($P = 0.0235$) as well as in NBQX-treated mice ($P = 0.0387$), compared to the UCMS vehicle group. The above results suggest a role for the AMPA receptor in the antidepressant-like effects of scopolamine combined with VU6001966.

4.1.9 Effects of the TrkB Receptor Antagonist ANA-12 on Scopolamine and VU6001966 Antidepressant Action

In the splash test, a two-way ANOVA revealed a significant interaction between ANA-12 (0.5 mg/kg) and a combination of scopolamine and VU6001966 [$F(1,27) = 17.03$, $P = 0.0003$; Fig. **11B**], along with a main effect of ANA-12 [$F(1,27) = 6.408$, $P = 0.0175$], but no significant main effect of tested combination [$F(1,27) = 3.844$, $P = 0.0603$]. Bonferroni's post hoc tests confirmed increased grooming time in group treated with given mixture compared to the UCMS vehicle group ($P = 0.0007$). In the SPT, no interaction between

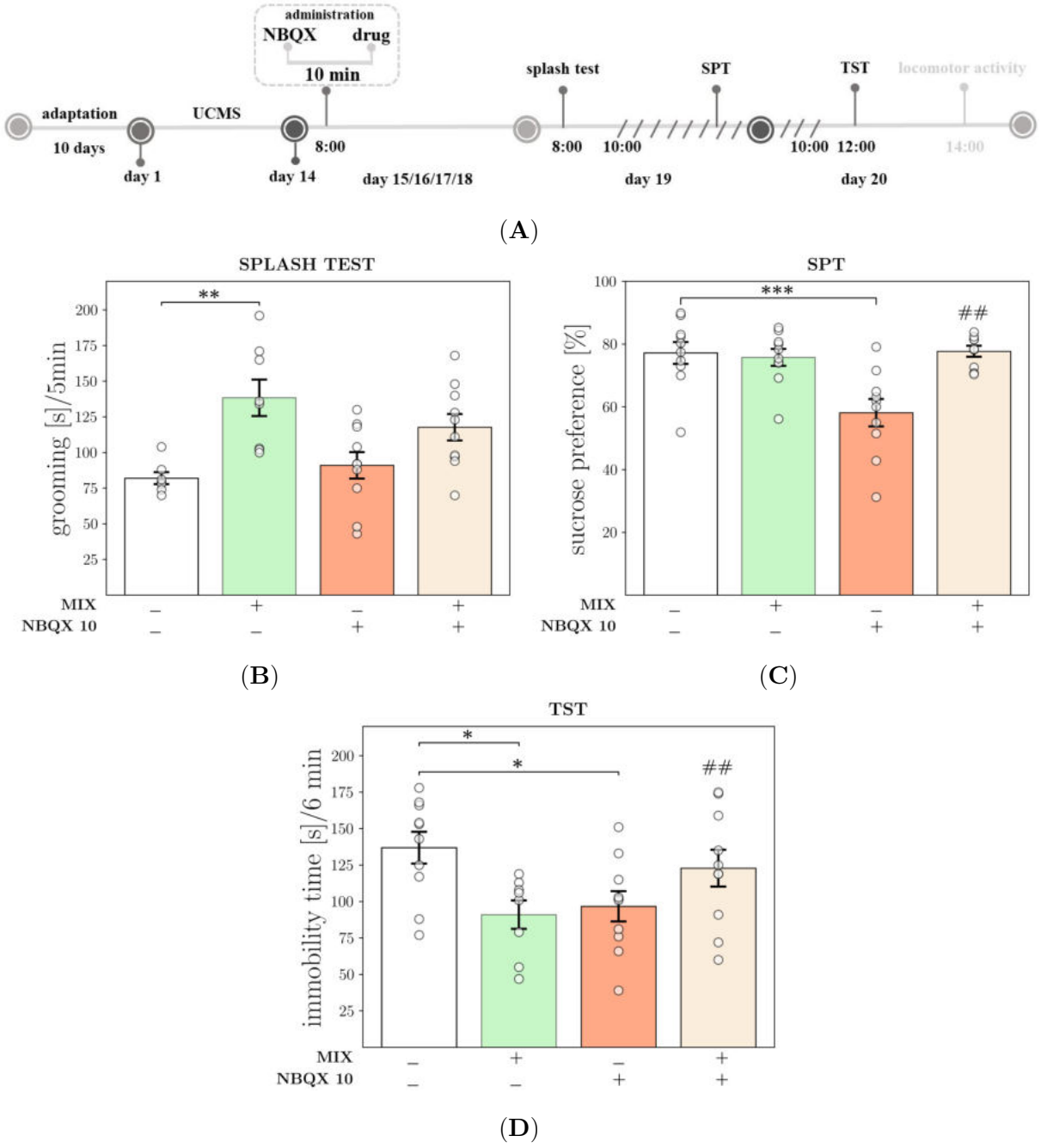


Figure 10: Behavioral effects of an AMPA receptor antagonist, NBQX, on the antidepressant-like action of scopolamine with VU6001966 in the UCMS model of depression. (A) Schematic representation of the experimental schedule. After a 10-day adaptation period to room conditions, the UCMS procedure was initiated and continued for 14 days. Beginning on day 15, the animals received subchronic treatment (four consecutive days) with either vehicle or the tested drugs. NBQX was injected 10 minutes before the mixture of scopolamine and VU6001966. Twenty-four hours after the last administration, the splash test was performed, followed two hours later by the SPT, which lasted for 24 hours. Two hours after completion of the SPT, the TST was conducted. Behavioral effects in (B) splash test, (C) SPT, (D) TST. Values are expressed as the mean ± SEM ($N = 7 - 10$) and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. respective vehicle; $\#\#P < 0.01$ interaction: scopolamine 0.1 mg/kg + VU6001966 1 mg/kg × NBQX). UCMS — unpredictable chronic mild stress; MIX — scopolamine 0.1 mg/kg + VU6001966 1 mg/kg.

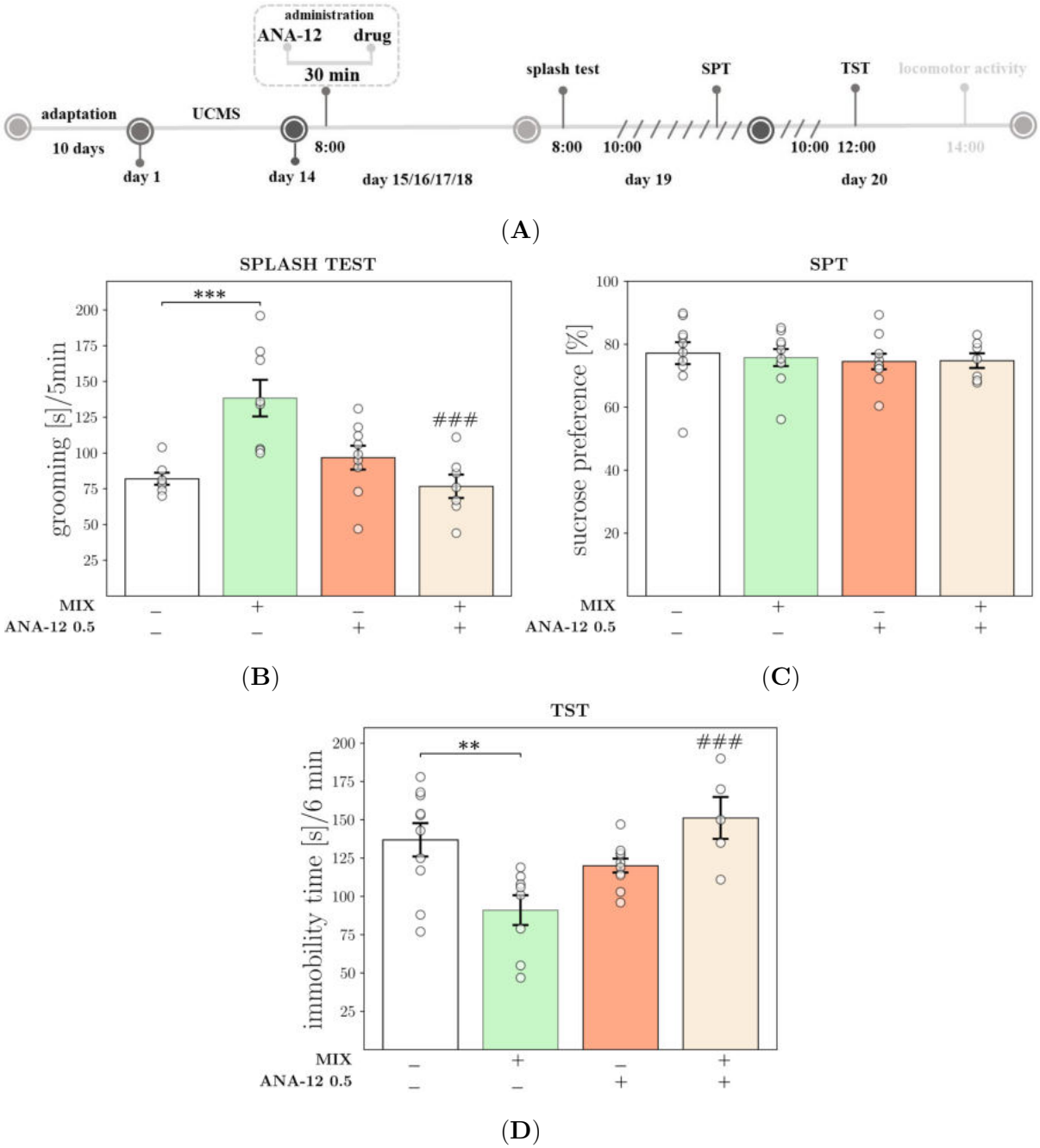
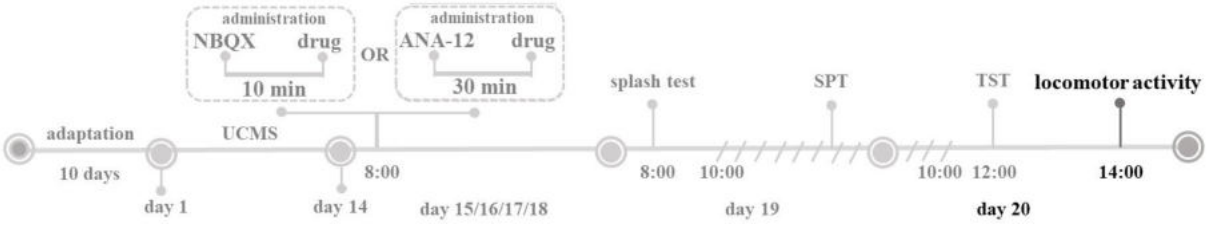


Figure 11: Behavioral effects of a TrkB receptor antagonist, ANA-12, on the antidepressant-like action of scopolamine with VU6001966 in the UCMS model of depression. (A) Schematic representation of the experimental schedule. After a 10-day adaptation period to room conditions, the UCMS procedure was initiated and continued for 14 days. Beginning on day 15, the animals received subchronic treatment (four consecutive days) with either vehicle or the tested drugs. ANA-12 was injected 30 minutes before the mixture of scopolamine and VU6001966. Twenty-four hours after the last administration, the splash test was performed, followed two hours later by the SPT, which lasted for 24 hours. Two hours after completion of the SPT, the TST was conducted. Behavioral effects in (B) splash test, (C) SPT, (D) TST. Values are expressed as the mean ± SEM ($N = 5 - 10$) and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test ($**P < 0.01$; $***P < 0.001$ vs. respective vehicle; $\#\#\#P < 0.001$ interaction: scopolamine 0.1 mg/kg + VU6001966 1 mg/kg × ANA-12). UCMS — unpredictable chronic mild stress; MIX — scopolamine 0.1 mg/kg + VU6001966 1 mg/kg.

ANA-12 and the combination of scopolamine and VU6001966 was observed [$F(1, 33) = 0.0852$, $P = 0.7722$; Fig. 11C], and neither the tested combination [$F(1, 33) = 0.03877$, $P = 0.8451$] nor ANA-12 [$F(1, 33) = 0.3848$, $P = 0.5393$] showed significant main effects. Bonferroni's multiple comparisons test confirmed no significant differences between groups ($P > 0.05$). In the TST, a significant interaction between ANA-12 and a combination of scopolamine and VU6001966 was observed in the splash test [$F(1, 29) = 15.25$, $P = 0.0005$; Fig. 11D], along with a main effect of ANA-12 [$F(1, 29) = 4.844$, $P = 0.0359$], but no significant main effect of tested combination [$F(1, 29) = 0.5633$, $P = 0.459$]. Bonferroni's post hoc tests confirmed decreased immobility in group treated with given mixture compared to the UCMS vehicle group ($P = 0.0039$). The above results suggest a role for the TrkB receptor in the antidepressant-like effects of scopolamine combined with VU6001966.

4.1.10 Effects of Tested Drugs on Locomotor Activity

To assess general activity and exclude any non-specific effects of the tested compounds that could interfere with the results of the TST, locomotor activity was measured. Two-way repeated measures ANOVA showed a significant interaction between time and drug treatment (combined administration and/or NBQX) [$F(12, 140) = 1.986$, $P = 0.0296$; Fig. 12B], along with a main time effect [$F(3, 167, 110.8) = 48.41$, $P < 0.0001$], but no significant main effect of tested drugs [$F(3, 35) = 2.544$, $P = 0.0719$]. Post hoc analysis with Bonferroni's multiple comparisons test indicated that none of the treatment groups differed significantly from the control group at any time point ($P > 0.05$). Two-way repeated measures ANOVA also showed a significant interaction between time and drug treatment (combined administration and/or ANA-12) [$F(12, 124) = 2.106$, $P = 0.0209$; Fig. 12C], along with a main time effect [$F(2, 565, 79.53) = 49.09$, $P < 0.0001$], but no significant main effect of tested drugs [$F(3, 31) = 2.357$, $P = 0.0909$]. Bonferroni's post hoc test did not show any significant differences at any tested time point ($P > 0.05$). Taken together, combined administration of scopolamine and VU6001966, as well as NBQX and ANA-12 — either alone or in combination with scopolamine and VU6001966 — did not affect mice's locomotor activity.



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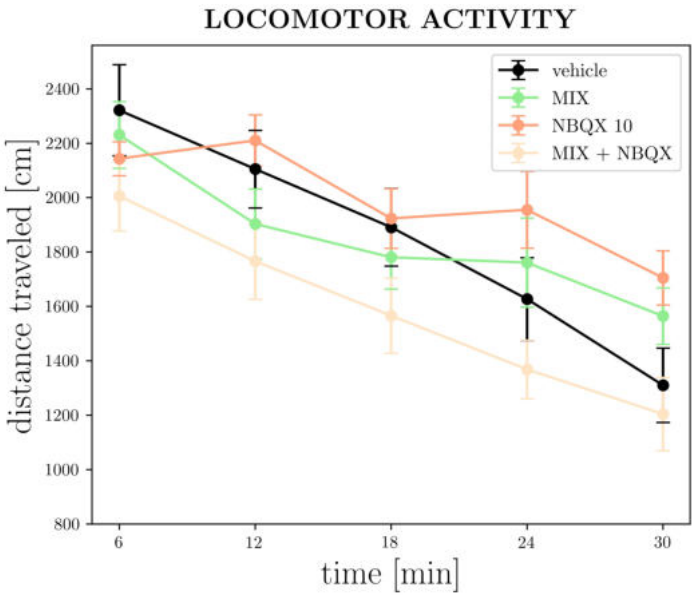

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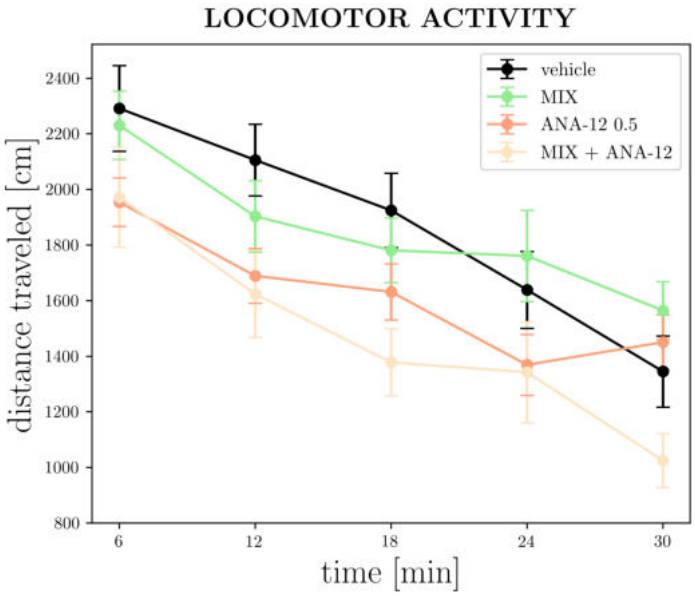

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Figure 12: Effects of the tested drugs on locomotor activity in C57BL/6J mice. (A) Schematic representation of the experimental schedule. Two hours after completion of the last behavioral test (TST), the locomotor activity test was performed for 30 minutes. Effects of the combination of scopolamine with VU6001966 (MIX): (B) with or without NBQX, and (C) with or without ANA-12, on locomotor activity. Values are expressed as the mean ± SEM ($N = 5 - 10$) and were analyzed by two-way repeated measurements ANOVA followed by Bonferroni's post hoc test. MIX — scopolamine 0.1 mg/kg + VU6001966 1 mg/kg.

4.1.11 Effects of Combined Scopolamine and VU6001966 on TrkB, eEF2, mTOR, and BDNF in the PFC

In the PFC, a one-way ANOVA revealed significant differences in the pTrkB/TrkB ratio among the groups [$F(4, 30) = 7.073$, $P = 0.0004$; Fig. **13A**]. Bonferroni post hoc test showed a significantly decreased pTrkB/TrkB ratio in stressed mice compared to non-stressed (NS) controls ($P = 0.0233$), as well as a reversal of these UCMS-induced changes by the tested mixture of scopolamine and VU6001966, referred to as 'MIX' ($P = 0.0492$). Statistical analyses of Western blots in the PFC showed no significant effects of the tested compounds on the levels of peEF2/eEF2 [$F(4, 30) = 2.528$, $P = 0.0612$; Fig. **13B**], pmTOR/mTOR [$F(4, 33) = 2.179$, $P = 0.093$; Fig. **13C**], or BDNF [$F(4, 30) = 2.638$, $P = 0.0533$; Fig. **13D**]. These results suggest that subchronic coadministration of scopolamine and VU6001966 significantly reversed the stress-induced decrease in the TrkB level in the PFC.

4.1.12 Effects of Combined Scopolamine and VU6001966 on TrkB, eEF2, mTOR, and BDNF in the Hippocampus

In the hippocampus, a one-way ANOVA revealed significant differences in the pTrkB/TrkB ratio among the groups [$F(4, 39) = 13.13$, $P < 0.0001$; Fig. **14A**]. Bonferroni post hoc analysis only indicated a significantly decreased pTrkB/TrkB ratio in the UCMS control group compared to the NS controls ($P = 0.0007$). No effects of the tested compounds were observed on the peEF2/eEF2 [$F(4, 32) = 2.113$, $P = 0.1022$; Fig. **14B**] or pmTOR/mTOR ratios [$F(4, 37) = 0.4292$, $P = 0.7865$; Fig. **14C**]. Although the ANOVA also showed statistically significant differences in BDNF levels [$F(4, 31) = 3.467$, $P = 0.0188$; Fig. **14D**], the post hoc test did not confirm changes between any specific groups ($P > 0.05$). Taken together, subchronic coadministration of scopolamine and VU6001966 did not affect the levels of the tested proteins in the hippocampus.

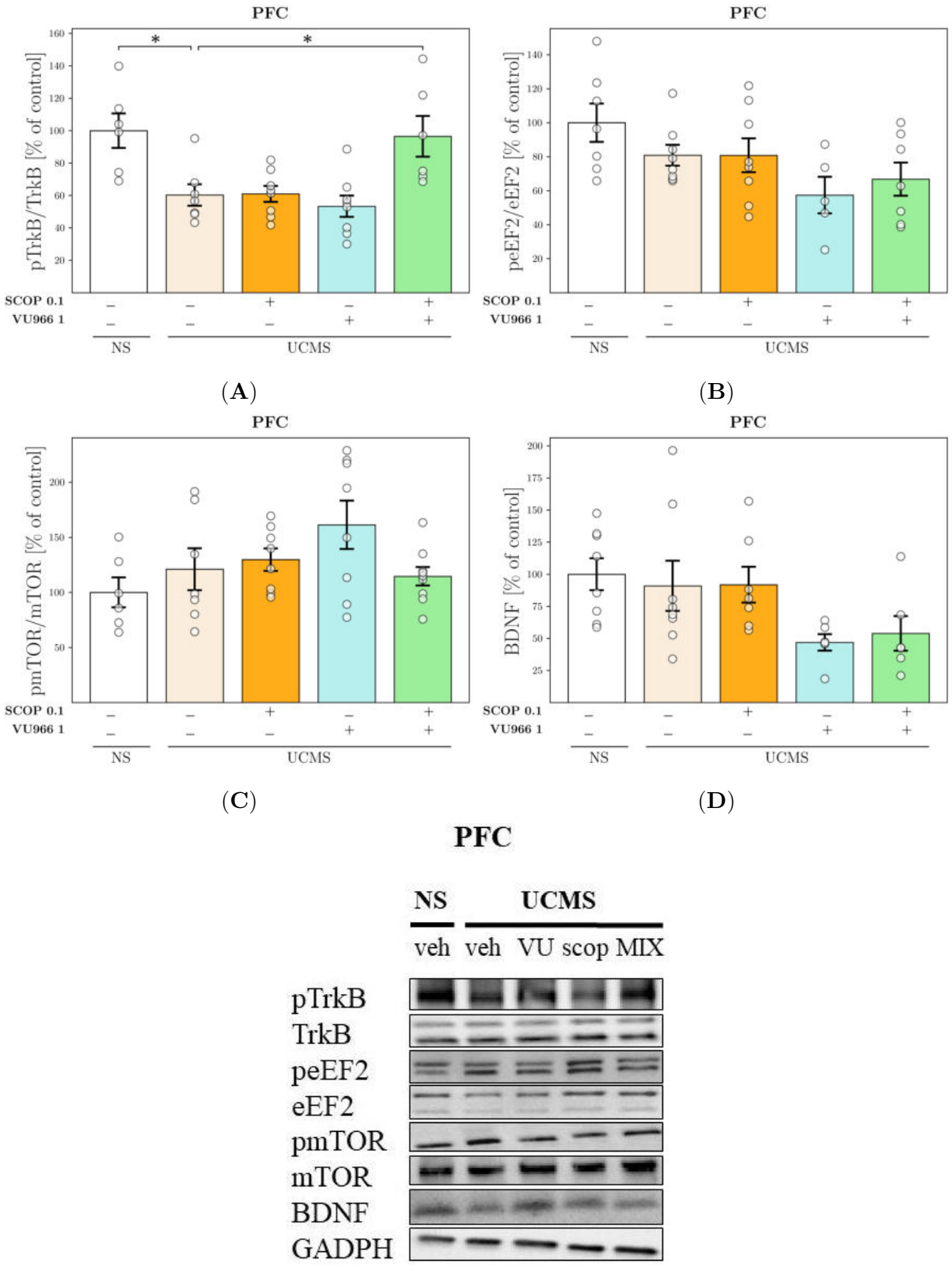


Figure 13: Effects of four-day coadministration of scopolamine with VU6001966 on the (A) pTrkB/TrkB, (B) peEF2/eEF2, (C) pmTOR/mTOR, and (D) BDNF protein levels analyzed by Western blot analysis in the PFC. The values are expressed as a percentage of changes vs. control levels ($N = 6 - 9$) and were analyzed by one-way ANOVA followed by Bonferroni's post hoc test ($*P < 0.05$ vs. respective vehicle). NS — non-stressed; UCMS — unpredictable chronic mild stress.

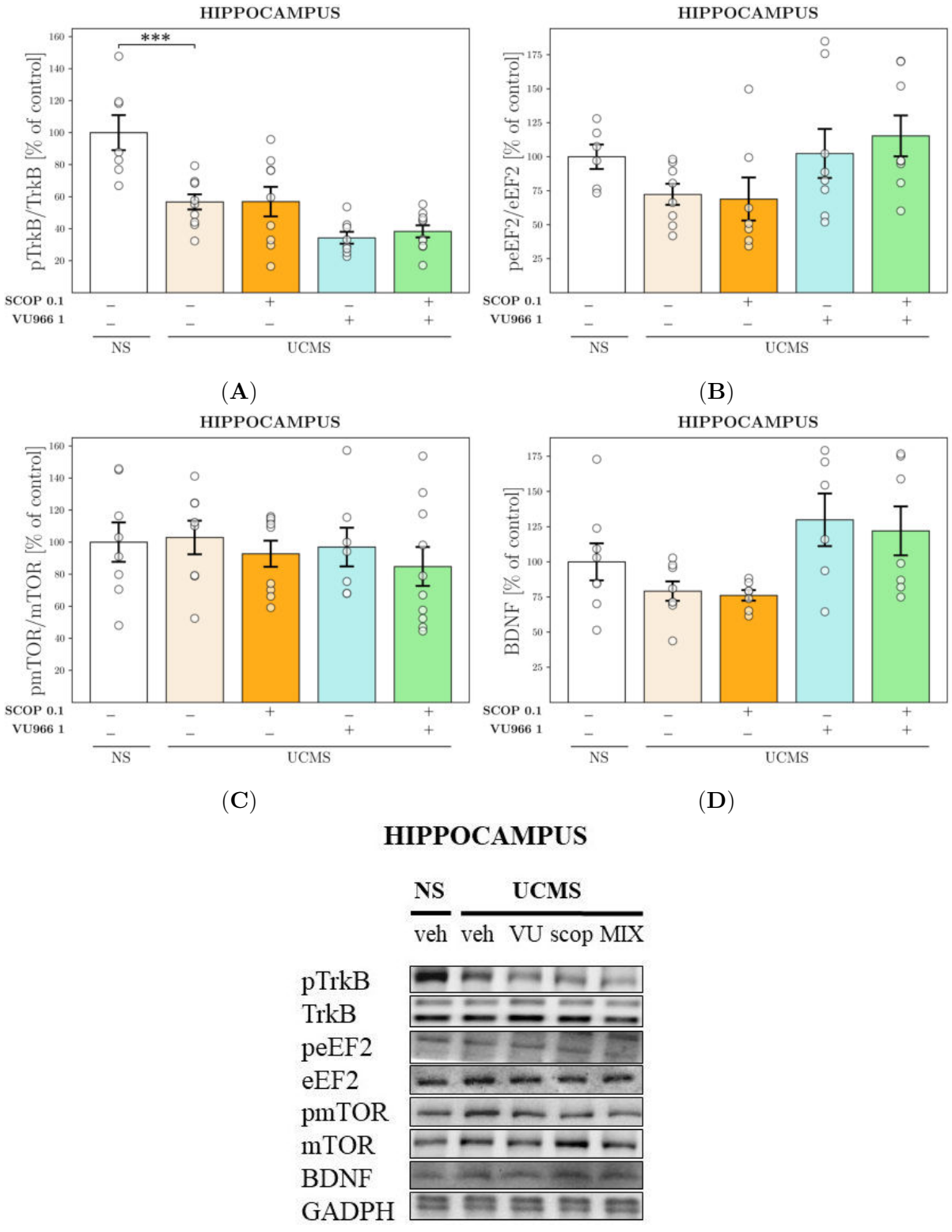


Figure 14: Effects of four-day coadministration of scopolamine with VU6001966 on the (A) pTrkB/TrkB, (B) peEF2/eEF2, (C) pmTOR/mTOR, and (D) BDNF protein levels analyzed by Western blot analysis in the hippocampus. The values are expressed as a percentage of changes vs. control levels ($N = 6 - 9$) and were analyzed by one-way ANOVA followed by Bonferroni's post hoc test ($***P < 0.001$ vs. respective vehicle). NS — non-stressed; UCMS — unpredictable chronic mild stress.

4.1.13 Cognitive Effects of Four-Day Scopolamine and VU6001966 Coadministration

To assess spatial memory, the object location test (OLT) was performed. Two-way ANOVA revealed no interaction between stress and the tested drugs [$F(4, 106) = 1.462$, $P = 0.219$; Fig. **15B**] and no main effect of stress [$F(1, 106) = 1.955$, $P = 0.165$], but a significant main effect of the tested drugs was observed [$F(4, 106) = 5.932$, $P = 0.0002$]. Bonferroni's post hoc test showed no significant differences between vehicle-treated UCMS mice and NS controls, as well as between vehicle-treated mice and mice treated with combination of scopolamine and VU6001966, in both naïve and stressed group ($P > 0.05$). Meanwhile, scopolamine given at referential dose of 1 mg/kg vividly decreased moved object investigation time ($P = 0.0051$ and $P = 0.0344$, for NS and UCMS group, respectively). The same effect was observed in the novel object recognition test (NORT) assessing non-spatial learning of object identity. No interaction between stress and tested drugs [$F(4, 101) = 1.069$, $P = 0.376$; Fig. **15C**] and no main effect of stress [$F(1, 101) = 1.502$, $P = 0.2233$] was indicated, however a significant effect of drugs on the tested parameter was observed [$F(4, 101) = 5.392$, $P = 0.0006$]. Bonferroni's multiple comparisons test showed no significant differences between vehicle-treated UCMS mice and NS controls, as well as between vehicle-treated mice and mice treated with combination of scopolamine and VU6001966, in both naïve and stressed group ($P > 0.05$). Meanwhile, scopolamine given at referential dose of 1 mg/kg significantly decreased novel object investigation time ($P = 0.0176$ and $P = 0.0466$, for NS and UCMS group, respectively). Taken together, these results indicate that subchronic coadministration of scopolamine and VU6001966 over four consecutive days did not impair spatial and non-spatial memory.

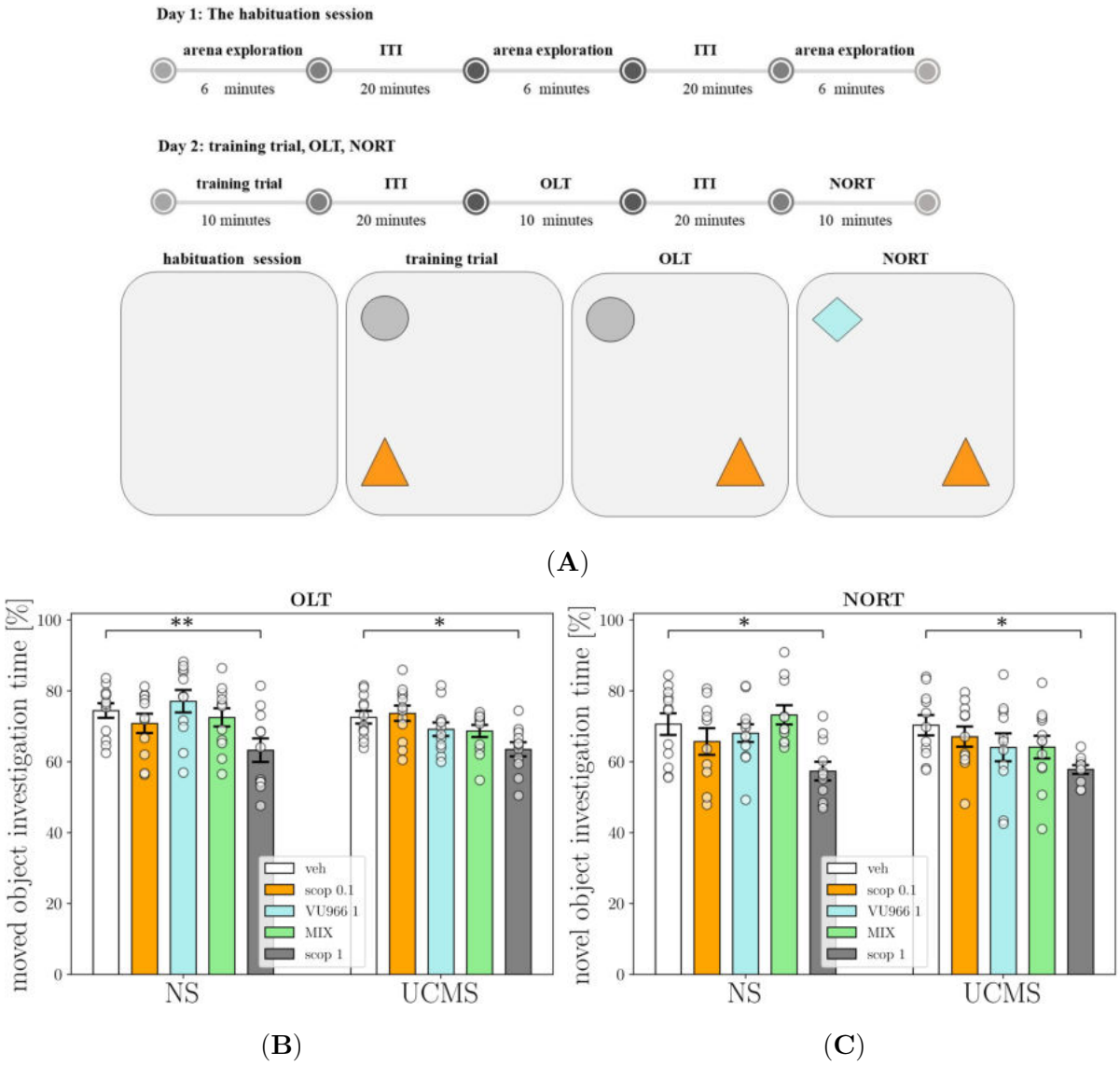


Figure 15: Cognitive effects of four-day coadministration of scopolamine with VU6001966 in the UCMS model of depression. (A) Schematic representation of the experimental schedule. During habituation, animals were placed in the arena without objects for 6 minutes, with two additional sessions following 20-minute intervals (3 total). Twenty-four hours later, they were exposed to two distinct objects for 10 minutes. After 20 minutes, the OLT was conducted by relocating one object. Another 20 minutes later, the NORT was performed by replacing the unmoved object with a novel one. The effect of the tested cotreatment on (B) the spatial working memory of the animals was measured by the OLT, and (C) the object recognition memory was measured by the NORT. Values are expressed as the mean ± SEM ($N = 5 - 10$) and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test ($** P < 0.01$; $*** P < 0.001$ vs. respective vehicle). ITI — inter-trial interval; NS — non-stressed; UCMS — unpredictable chronic mild stress; MIX — scopolamine 0.1 mg/kg + VU6001966 1 mg/kg.

4.2 Studies Conducted on Rats

4.2.1 Antidepressant-Like Effects of Scopolamine

One-way ANOVA revealed dose-dependent effects of scopolamine ($0.03 - 0.3$ mg/kg, IP), administered 45 minutes before the FST, on immobility time [$F(3, 27) = 5.85$, $P = 0.003$; Fig. **16B**]. Dunnett's post hoc tests indicated that scopolamine significantly decreased immobility time at doses of 0.1 and 0.3 mg/kg ($P = 0.020$ and $P = 0.002$, respectively). Scopolamine also affected climbing time [$F(3, 28) = 11.22$, $P < 0.0001$; Fig. **16C**], with Dunnett's post hoc tests showing a significant increase at 0.3 mg/kg ($P < 0.0001$). Scopolamine also affected swimming time [$F(3, 27) = 4.72$, $P = 0.009$; Fig. **16D**], with significant increases observed at 0.1 mg/kg ($P = 0.009$) and 0.3 mg/kg ($P = 0.043$). Taken together, these results indicate that acute scopolamine treatment exerted antidepressant-like effects in the FST in Sprague Dawley rats. At a dose of 0.03 mg/kg, scopolamine had no significant effect on any measured parameter.

4.2.2 Antidepressant-Like Effects of VU6001966

A one-way ANOVA revealed that VU6001966 ($1 - 10$ mg/kg, IP) administered 45 min before the FST significantly affected immobility time [$F(3, 25) = 3.06$, $P = 0.047$; Fig. **17B**], with Dunnett's post hoc tests showing a significant decrease at 10 mg/kg ($P = 0.037$). VU6001966 had no impact on climbing [$F(3, 26) = 2.00$, $P = 0.139$; Fig. **17C**] or swimming time [$F(3, 26) = 2.55$, $P = 0.078$; Fig. **17D**]. Taken together, these results indicate that acute VU6001966 treatment exerted antidepressant-like effects in the FST in Sprague Dawley rats. When applied at doses of 1 and 3 mg/kg, VU6001966 had no effect on the tested parameters.

4.2.3 Antidepressant-Like Effects of Combined Scopolamine and VU6001966

To study the acute antidepressant-like effects of combined administration in rats, the subeffective doses of 0.03 and 3 mg/kg for scopolamine and VU6001966, respectively, were selected for the experiment.

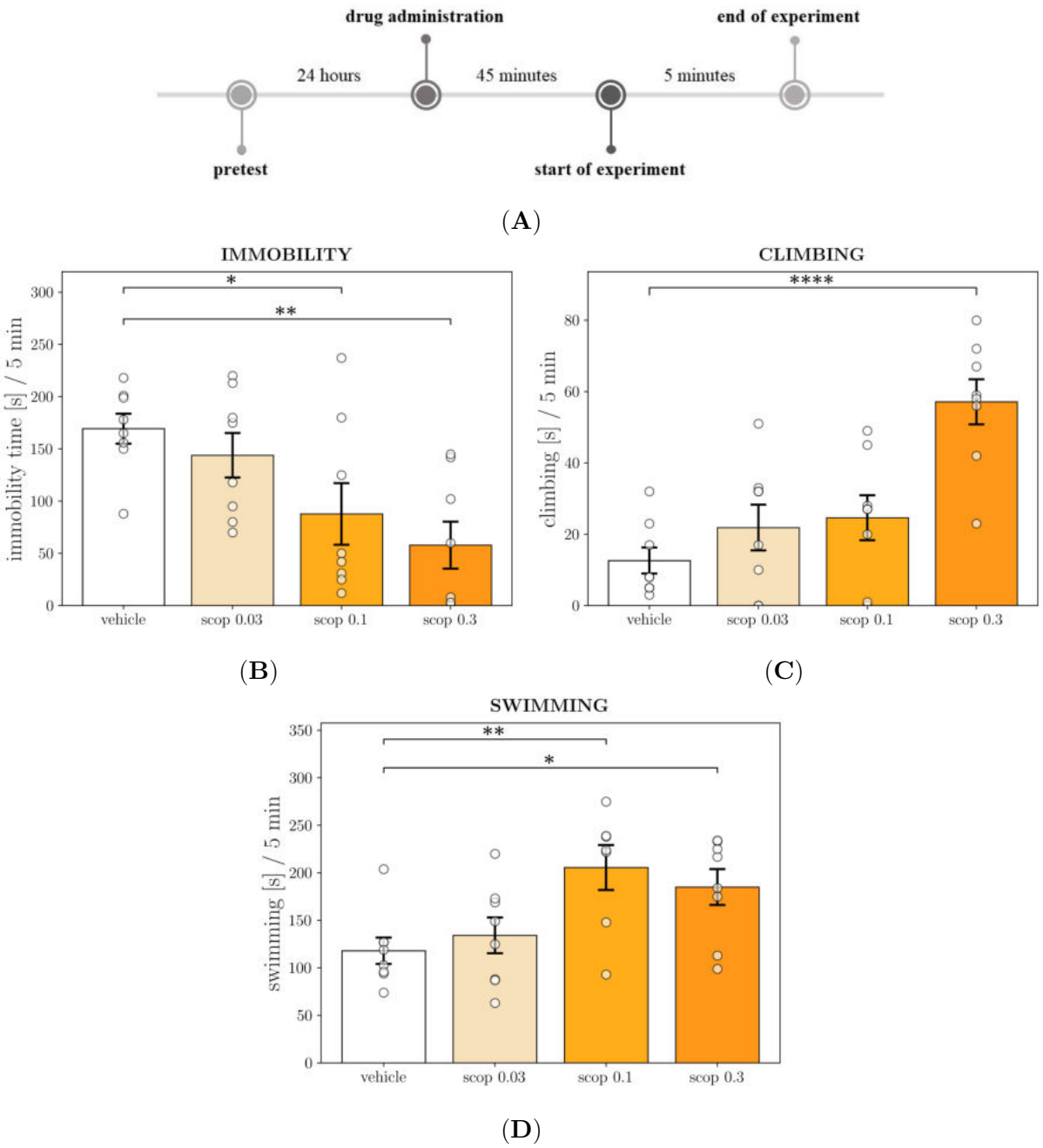


Figure 16: The antidepressant-like effects of scopolamine administered IP 45 minutes before the FST in Sprague Dawley rats. (A) Schematic representation of the experimental schedule. The following three parameters were measured: (B) immobility, (C) climbing, and (D) swimming. Values are expressed as the mean ± SEM ($N = 7 - 8$) and were analyzed by one-way ANOVA followed by Dunnett's post hoc test ($*P < 0.05$; $**P < 0.01$; $****P < 0.0001$ vs. respective vehicle).

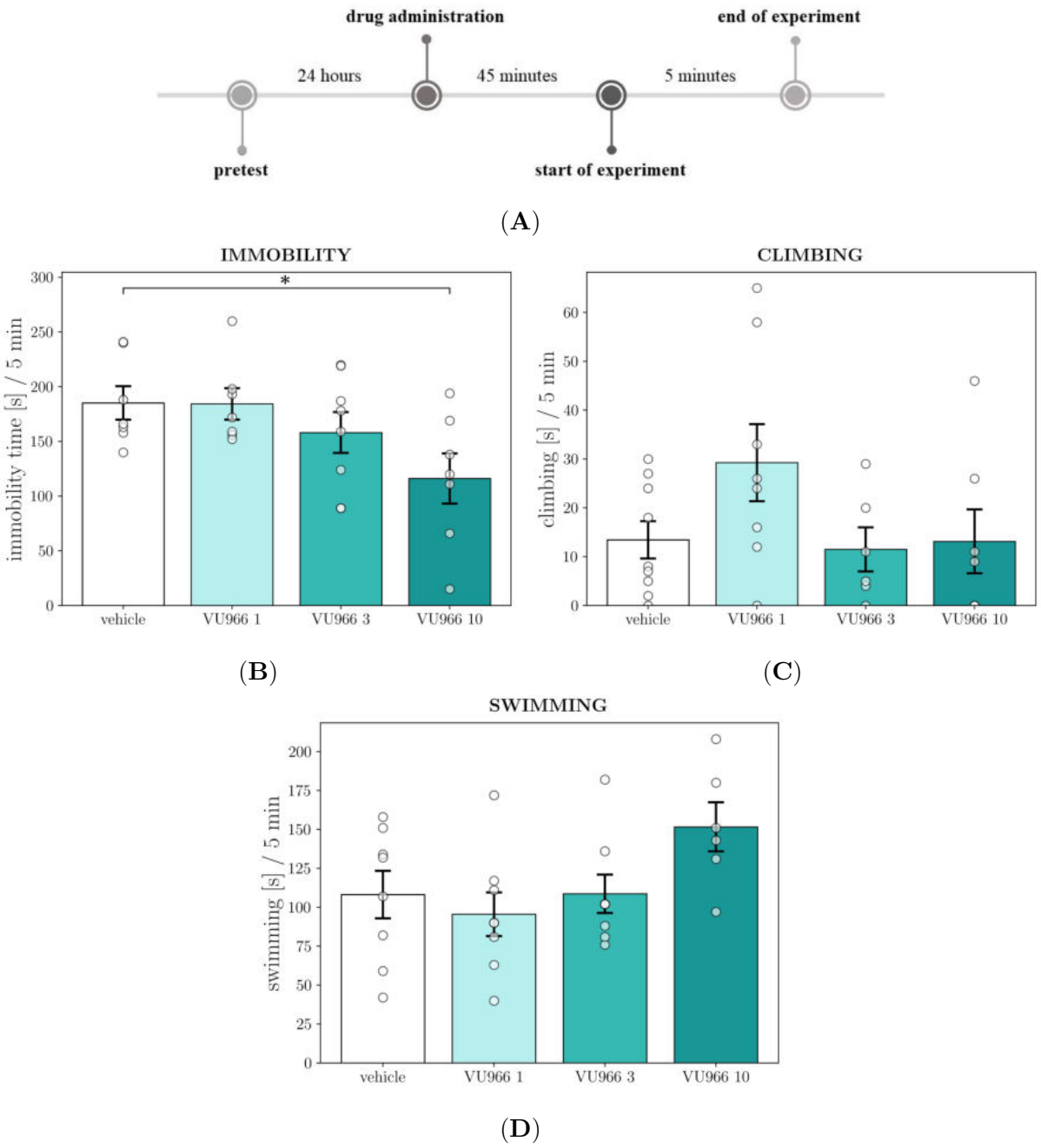


Figure 17: The antidepressant-like effects of VU6001966 administered IP 45 minutes before the FST in Sprague Dawley rats. (A) Schematic representation of the experimental schedule. The following three parameters were measured: (B) immobility, (C) climbing, and (D) swimming. Values are expressed as the mean ± SEM ($N = 7 - 8$) and were analyzed by one-way ANOVA followed by Dunnett's post hoc test ($*P < 0.05$ vs. respective vehicle).

Two-way ANOVA revealed that combined administration of scopolamine (0.03 mg/kg) and VU6001966 (3 mg/kg), given 45 minutes before the FST, induced a significant decrease of immobility [$F(1, 22) = 4.35$, $P = 0.049$; Fig. 18B] and increase of climbing time [$F(1, 26) = 11.00$, $P = 0.003$; Fig. 18C], while no affecting the swimming time

[$F(1, 28) = 0.13$, $P = 0.726$; Fig. 18D]. These results indicate that a subeffective dose of VU6001966 significantly enhanced the acute antidepressant-like effects of a subeffective dose of scopolamine in the FST in rats.

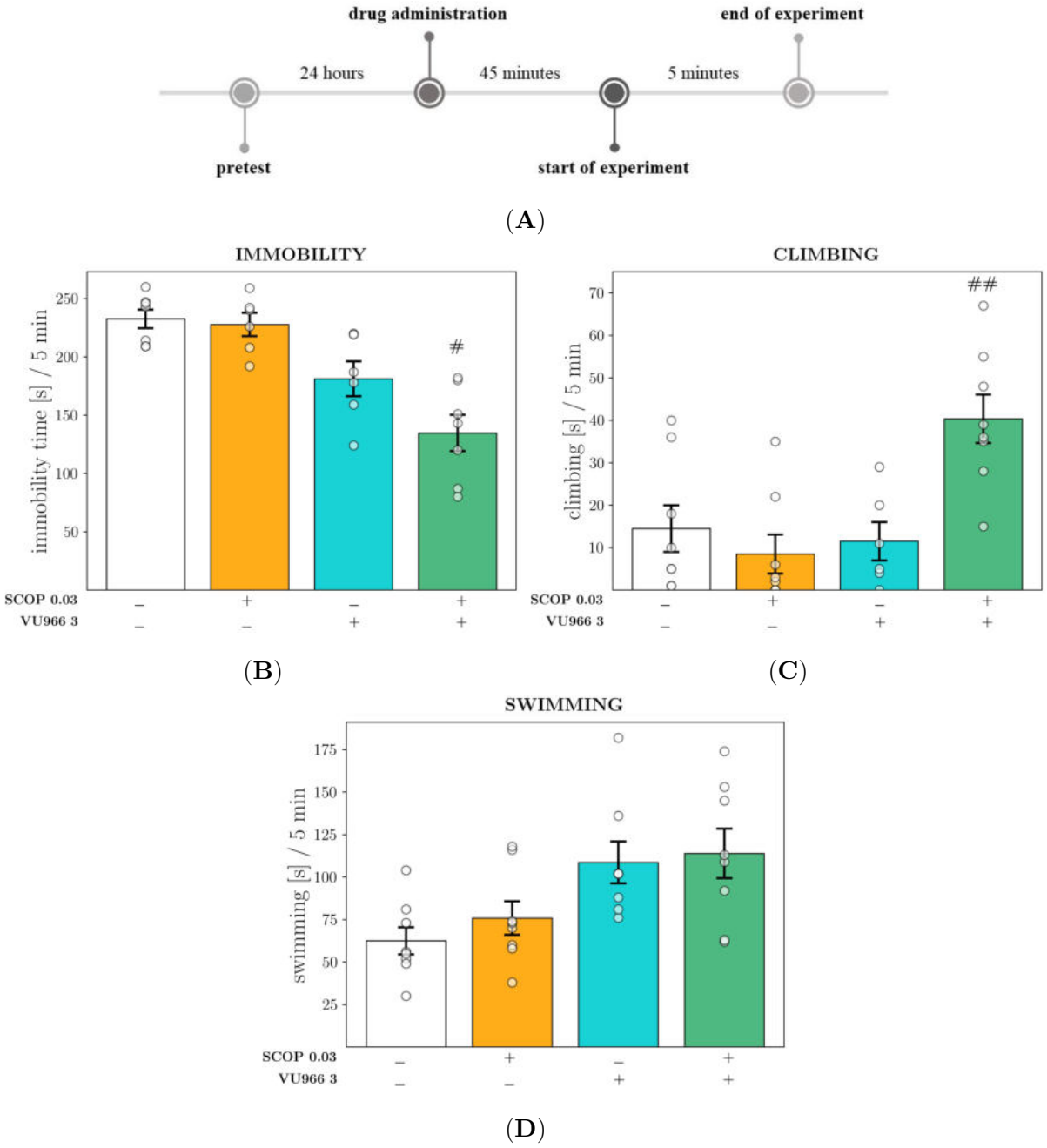


Figure 18: The antidepressant-like effects of scopolamine and VU6001966 coadministered IP 45 minutes before the FST in Sprague Dawley rats. (A) Schematic representation of the experimental schedule. The following three parameters were measured: (B) immobility, (C) climbing, and (D) swimming. Values are expressed as the mean ± SEM ($N = 7 - 8$) and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test ($\#P < 0.05$, $\#\#P < 0.01$; interaction: scopolamine × VU6001966).

4.2.4 Effects of Scopolamine and VU6001966 on Extracellular 5-HT, DA, Glutamate, and GABA in the Frontal Cortex

Treatment with either scopolamine (0.3 mg/kg) or VU6001966 (3 mg/kg) alone elevated 5-HT levels to approximately 300% of baseline, whereas combined administration increased levels up to 400% (Fig. **19B**). Repeated-measures ANOVA revealed a significant effect of treatment group ($F(3, 17) = 218.45$, $P < 0.0001$), as well as a significant interaction between treatment groups and sampling period ($F(15, 85) = 30.71$, $P < 0.0001$). A two-way ANOVA revealed a significant interaction between scopolamine (0.3 mg/kg) and VU6001966 (3 mg/kg) in their total effects on 5-HT levels, expressed as AUC [$F(1, 17) = 14.83$, $P = 0.001$; Fig. **19C**]. Tukey's post hoc tests indicated that both compounds, alone or in combination, significantly increased 5-HT levels compared with controls ($P < 0.0001$). Similarly, scopolamine (0.3 mg/kg) and VU6001966 (3 mg/kg) each significantly increased extracellular DA levels to 200% of baseline in the rat frontal cortex, with combined administration further enhancing DA to 400% of baseline (Fig. **19D**). Repeated-measures ANOVA showed the significant effect of treatment groups ($F(3, 20) = 38.66$, $P < 0.0001$) and the interaction between treatment groups and sampling period ($F(15, 100) = 9.25$, $P < 0.0001$). A two-way ANOVA revealed a significant interaction between scopolamine (0.3 mg/kg) and VU6001966 (3 mg/kg) in their total effects on DA levels, expressed as AUC [$F(1, 20) = 4.86$, $P = 0.039$; Fig. **19E**]. Both compounds, alone or in combination, significantly increased DA levels compared with controls ($P < 0.0001$). Extracellular glutamate levels in the rat frontal cortex were increased by scopolamine (0.3 mg/kg) to 150% of baseline and more potently by the combination of scopolamine and VU6001966 to 220% of baseline, whereas VU6001966 alone (3 mg/kg) decreased glutamate levels (Fig. **20B**). Repeated-measures ANOVA showed the significant effect of treatment groups ($F(3, 22) = 193.84$, $P < 0.0001$) and the interaction between treatment groups and sampling period ($F(15, 110) = 24.46$, $P < 0.0001$). A two-way ANOVA revealed a significant interaction between scopolamine (0.3 mg/kg) and VU6001966 (3 mg/kg) in their total effects on glutamate levels, expressed as AUC [$F(1, 22) = 135.60$, $P < 0.0001$; Fig. **20C**].

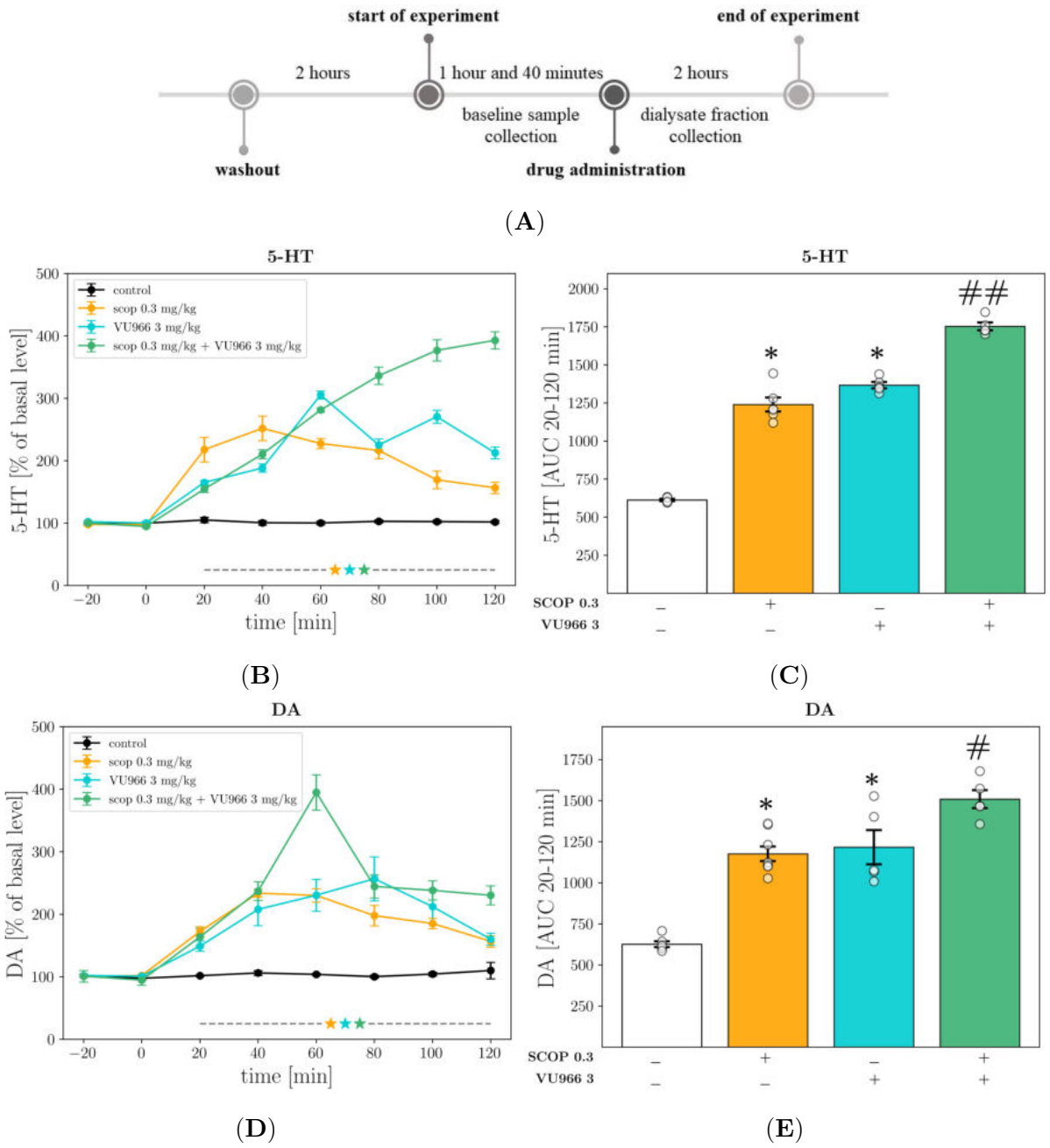


Figure 19: Effects of scopolamine (0.3 mg/kg), VU6001966 (3 mg/kg), and their combination on extracellular monoamine levels in the frontal cortex of Sprague Dawley rats. (A) Schematic representation of the experimental schedule. The time-course (B, D) and total effect (C, E) of tested compounds on serotonin (5-HT) and dopamine (DA) extracellular levels in the rat frontal cortex. The drug injection corresponds to time 0. The statistical significance of time curves, compared to the control, within the time range of 20–120 minutes is indicated by dashed lines, with stars colored to match the corresponding curve. The total effect is calculated as an area under the concentration-time curve (AUC) and expressed as a percentage of the basal level. Values are expressed as the mean ± SEM ($N = 5 - 8$) and were analyzed by two-way repeated measures ANOVA on normalized responses followed by Tukey's post hoc test (time course) and a two-way ANOVA followed by Bonferroni's post hoc test (total effect). $*P < 0.001$ vs. respective vehicle; $\#P < 0.05$, $\#\#P < 0.01$; interaction: scopolamine × VU6001966.

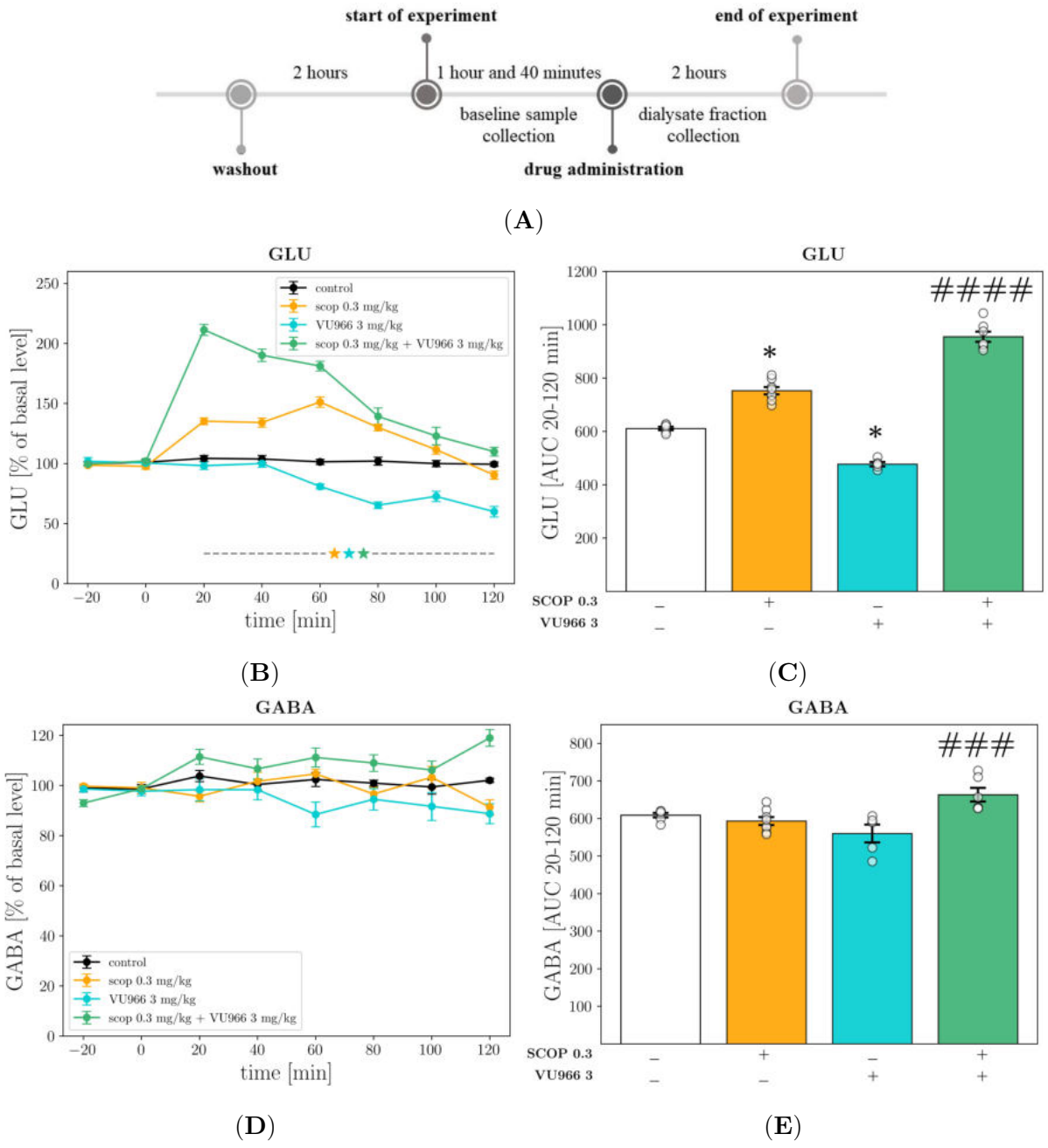
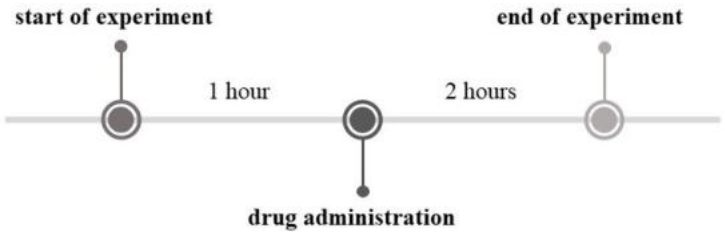


Figure 20: Effects of scopolamine (0.3 mg/kg), VU6001966 (3 mg/kg), and their combination on extracellular levels of amino acids in the frontal cortex of Sprague Dawley rats. (A) Schematic representation of the experimental schedule. The time-course (B, D) and total effect (C, E) of tested compounds on glutamate (GLU) and γ-aminobutyric acid (GABA) extracellular levels in the rat frontal cortex. The drug injection corresponds to time 0. The statistical significance of time curves, compared to the control, within the time range of 20–120 minutes is indicated by dashed lines, with stars colored to match the corresponding curve. The total effect is calculated as an area under the concentration-time curve (AUC) and expressed as a percentage of the basal level. Values are expressed as the mean ± SEM ($N = 5 - 8$) and were analyzed by two-way repeated measures ANOVA on normalized responses followed by Tukey's post hoc test (time course) and a two-way ANOVA followed by Bonferroni's post hoc test (total effect). $*P <$ 0.001 vs. respective vehicle; $\#\#\#P < 0.001$, $\#\#\#\#P < 0.0001$; interaction: scopolamine × VU6001966.

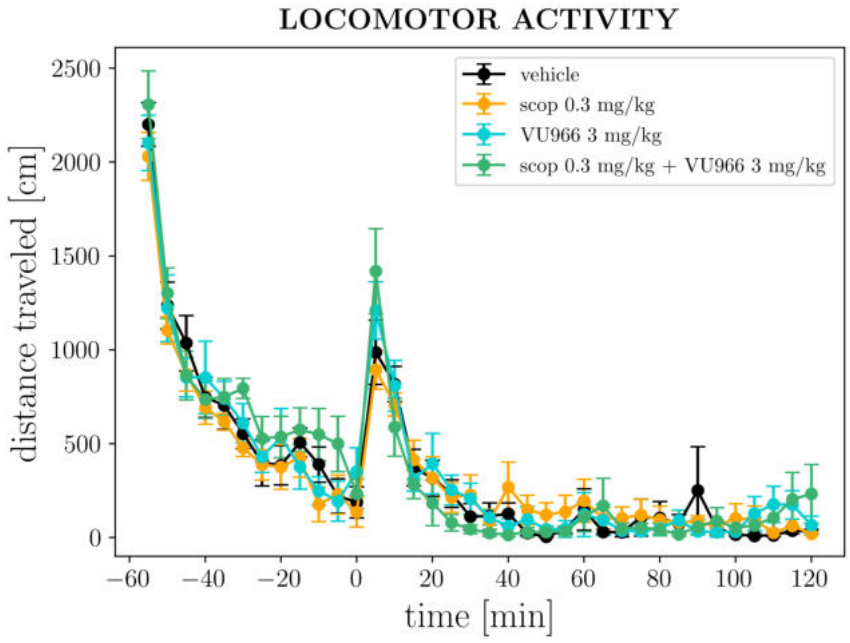
Post hoc analysis confirmed that both compounds, alone or in combination, significantly altered glutamate levels compared with controls ($P < 0.0001$). In contrast, none of the tested drugs affected extracellular GABA levels in the frontal cortex (Fig. **20D**). Repeated-measures ANOVA showed the significant effect of treatment groups ($F(3, 21) = 7.76$, $P = 0.001$) and the interaction between treatment groups and sampling period ($F(15, 105) = 3.86$, $P < 0.0001$). A two-way ANOVA revealed a significant interaction between scopolamine (0.3 mg/kg) and VU6001966 (3 mg/kg) in their total effects on GABA levels, expressed as AUC [$F(1, 21) = 15.86$, $P = 0.001$; Fig. **20E**]. However, post hoc tests revealed no significant differences compared with controls ($P > 0.05$). To sum up, acute treatment with scopolamine (0.3 mg/kg) and VU6001966 (3 mg/kg), alone or in combination, significantly increased extracellular 5-HT and DA levels in the rat frontal cortex, with combined administration producing the greatest enhancement. Glutamate was increased by scopolamine and even more by the combination, whereas VU6001966 alone slightly decreased glutamate levels. None of the treatments significantly altered extracellular GABA.

4.2.5 Effects of Tested Drugs on Spontaneous Locomotor Activity

To eliminate non-specific effects of the tested compounds that could influence FST immobility measurements and to assess whether dopaminergic activation in the frontal cortex affected rat mobility, locomotor activity was measured. Scopolamine, VU6001966, and a combination of these drugs were administered at doses used in microdialysis. No significant differences were observed between control rats and those treated with scopolamine (0.3 mg/kg) [$F(1, 13) = 0.04$, $P = 0.853$], VU6001966 (3 mg/kg) [$F(1, 14) = 0.06$, $P = 0.807$], or the mixture of these drugs [$F(1, 14) = 0.22$, $P = 0.648$] (Fig. **21B**). Taken together, these results indicate that scopolamine and VU6001966, whether administered alone or in combination, did not affect locomotor activity in rats.



(**A**)



(**B**)

Figure 21: Effects of scopolamine, VU6001966, and both in combination on the locomotor activity in Sprague Dawley rats. (A) Schematic representation of the experimental schedule. (B) The effect of a single administration of tested drugs on the spontaneous locomotor activity of rats during a 180-minute experimental session. The drug injection corresponds to time 0. The values are expressed as the mean ± SEM ($N = 7 - 8$) and were analyzed by two-way repeated measurements ANOVA followed by Bonferroni's post hoc test.

Chapter 5

Discussion

Major depressive disorder is a highly heterogeneous condition, and current translational models often fail to capture its full complexity, partly due to limited cross-species relevance. As a result, the biological basis of MDD — and consequently its treatment and prevention — remains only partially understood. Despite these challenges, growing data from animal research have enabled the depressive phenotypes to be translated into biologically more tractable dimensions, offering the potential for clinical translation. Rodent models of depression remain a valuable tool for identifying and validating new therapeutic targets and for investigating the mechanisms that underlie their antidepressant efficacy.

5.1 Evaluating Depressive-Like Phenotypes in Mice via the UCMS Paradigm

To assess antidepressant-like effects and elucidate the mechanism of action of the tested compounds, the unpredictable chronic mild stress (UCMS) model in mice was used. This widely used chronic stress paradigm not only allows measurement of parameters reflecting core symptoms of depression but also allows reliable estimation of the time required for a compound to produce therapeutic effects in humans, enabling differentiation between classical and rapid-acting antidepressants (Willner, 2017). These parameters included: reduced grooming in the splash test (a measure of apathy), decreased sucrose preference (reflecting anhedonia), and increased immobility in the TST and FST (indicative of behavioral despair). When designing an experimental scheme for the UCMS model with the goal of achieving high reproducibility, it is essential to carefully consider multiple

variables. These include the type and duration of stressors applied, as well as the animal strain selected. To maintain unpredictability and prevent adaptation, the same stressor should not be used on consecutive days. Additionally, stressors must be appropriately matched, neither too mild to be ineffective nor excessively intense, as the latter may induce health complications that confound results. In this study, severe stressors such as prolonged food or water deprivation and painful stressors were intentionally excluded, as they do not accurately reflect the etiology of depression in humans and may produce generalized physiological effects that interfere with the measures of the UCMS-induced effects (Markov & Novosadova, 2022). Another important consideration is the circadian activity of rodents; since mice are nocturnal, the application of stressors during the light phase may induce chronic sleep disruption, serving as an additional stressor that should be accounted for (Murack et al., 2021). The C57BL/6J mouse strain was chosen for this study due to its known high susceptibility to UCMS and responsiveness to antidepressant treatments. However, it is worth noting that this strain may not be suitable for extended UCMS protocols, as it can develop adaptive responses that diminish stress-induced effects over time (Pałucha-Poniewiera et al., 2020). This limitation was not a concern in the present study, as the UCMS protocol was limited to 14 days. Furthermore, to ensure consistency across experimental groups, animals of the same age were used throughout, given that age-related differences in stress sensitivity, neuroplasticity, and pharmacological responsiveness can significantly influence outcomes. The results presented in this dissertation indicate that the UCMS protocol effectively induced depressive-like behavior, as evidenced by reduced grooming time in the splash test and decreased sucrose preference in the SPT. However, the expected stress-induced increase in immobility in the TST or FST was not consistently observed (Fig. **3D**, **3E**, **9D**). Although not statistically significant, vehicle-treated UCMS mice showed a trend toward reduced immobility compared to stress-naïve controls. This apparent increase in escape-oriented behavior among stressed mice may reflect the development of anxiety-like hyperactivity by chronic stress, rather than passive immobility (Anyan & Amir, 2018; Tran & Gellner, 2023; Venzala et al., 2013).

5.2 Behavioral Effects of Single Versus Subchronic Administration of Scopolamine in the UCMS Model of Depression

Scopolamine has demonstrated rapid-acting antidepressant effects in patients with MDD (Ellis et al., 2014; Furey & Drevets, 2006; Furey et al., 2010). However, preclinical evidence supporting these findings remains limited. To date, only one study has directly examined the onset of scopolamine's action in an animal model. In this work, a single intraperitoneal dose of scopolamine (0.25 or 0.5 mg/kg) significantly reduced escape failures in the learned helplessness model of depression in male Sprague Dawley rats, whereas the tricyclic antidepressant imipramine required chronic administration to achieve similar effects (M. E. Ballard et al., 2007). Building on this, the first objective of the present study was to compare the antidepressant-like effects of single versus subchronic administration of scopolamine in the UCMS model of depression. The four-day administration schedule used in the present study was based on the regimen applied in studies of the muscarinic receptor antagonist penehyclidine, which demonstrated antidepressant potential in humans (Sun et al., 2019). Our results revealed that subchronic treatment with scopolamine is the minimal but sufficient requirement for alleviating stress-induced depressive-like behaviors and for eliciting rapid and sustained antidepressant-like effects. Specifically, scopolamine administered at 0.3 mg/kg for four consecutive days significantly increased grooming time in the splash test (Fig. **4B**) and sucrose preference in the SPT (Fig. **4C**), while also reducing immobility in both the TST (Fig. **4D**) and FST (Fig. **4E**), in comparison to stress-naïve control animals. In contrast, a single administration at the same dose failed to reverse these UCMS-induced symptoms (Fig. **3B–3E**). Importantly, the observed antidepressant-like effects of subchronic scopolamine persisted for at least three days following the last injection, as evidenced by behavioral testing conducted during this period. This suggests that scopolamine may initiate longer-lasting neuroadaptive processes underlying its efficacy. Many animal studies assessing scopolamine's antidepressant-like properties focus solely on its acute effects, typically evaluating behavior within one hour of administration. Such an approach does not capture potential sustained effects, which are likely to involve the initiation of synaptic plasticity mechanisms. Our findings demonstrate

that a single dose of scopolamine is insufficient to trigger prolonged antidepressant-like effects, underlying the importance of subchronic treatment paradigms. Moreover, subchronic administration of scopolamine effectively reverses UCMS-induced depressive-like behavior without altering the behavior of non-stressed animals. This aligns with clinical evidence showing that scopolamine improves symptoms in depressed patients but has minimal impact on healthy individuals. Scopolamine acts as a nonselective muscarinic cholinergic receptor antagonist. The involvement of acetylcholine in the pathophysiology of depression is well-established (Dulawa & Janowsky, 2019). Acetylcholine modulates the balance between sympathetic "fight-or-flight" responses and parasympathetic "rest-and-digest" processes. Stress, a key trigger for depression, disrupts this balance, and elevated acetylcholine levels have been reported in depressed patients (Dulawa & Janowsky, 2019). On the other hand, studies in unstressed rats have shown that scopolamine administration increases acetylcholine release in both the frontal cortex and hippocampus (Toide, 1989). Together, these findings suggest that scopolamine's efficacy in reversing depressive-like behavior is specific to stressed animals, likely due to an upregulated cholinergic system.

5.3 Effects of Scopolamine on mTOR, eEF2, TrkB, and PSD95 in the PFC and Hippocampus in the UCMS Model of Depression

We examined molecular changes in the PFC and hippocampus — brain regions consistently implicated in the pathophysiology of MDD. The PFC is critical for cognitive control and emotional regulation, whereas the hippocampus, as part of the limbic system, is tightly interconnected with cognitive and behavioral networks, including the PFC. Converging evidence indicates that MDD is associated with neuronal atrophy and synaptic dysfunction in these regions (Belleau et al., 2019; Kempton et al., 2011). Based on this, we examined whether the antidepressant-like mechanisms of scopolamine involve signaling pathways within the PFC and hippocampus. Previous studies have highlighted the role of mammalian target of rapamycin (mTOR), brain-derived neurotrophic factor (BDNF)-tropomyosin receptor kinase B (TrkB), and eukaryotic elongation factor 2 (eEF2) signaling in regulating synaptogenesis and mediating the antidepressant effects of the rapid-acting

agent ketamine (Autry et al., 2011; N. Li et al., 2010; W. Zhou et al., 2014). Additionally, postsynaptic density protein 95 (PSD-95), through its interaction with TrkB in regulating BDNF signaling, has been suggested as a potential antidepressant target (Shi et al., 2024). To explore this further, we assessed protein levels of TrkB, mTOR, eEF2, and PSD-95 in the PFC and hippocampus following subchronic scopolamine administration. Our results revealed that subchronic treatment of scopolamine significantly altered phosphorylation of eEF2 at Thr56 in the PFC (Fig. **5A**). eEF2 is an essential factor for protein synthesis, which regulates translation elongation (Ryazanov et al., 1988). Its phosphorylation suppresses protein synthesis and synaptic plasticity, whereas dephosphorylation promotes BDNF translation and initiates plasticity-related processes, playing a pivotal role in the rapid antidepressant action of ketamine (Krystal et al., 2024; Taha et al., 2013). We found that chronic stress increased the peEF2/eEF2 ratio, thereby suppressing eEF2 activity, while scopolamine treatment reversed this effect. These findings suggest that eEF2 dephosphorylation in the PFC may contribute to the prolonged antidepressant-like effect of scopolamine. Interestingly, these effects were not observed in the hippocampus, where only a nonsignificant trend was observed (Fig. **6A**). Such regional differences may relate to systems-level memory consolidation processes. Following stress or learning, memory traces are initially encoded in the hippocampus but subsequently stabilized in cortical regions, such as the PFC, while hippocampal traces diminish over time (Goto, 2022). Given the involvement of eEF2 in hippocampus-dependent cognition (Gosrani et al., 2020), its phosphorylation state may vary with stress duration and timing.

Western blot analysis further showed no significant changes in PSD-95 levels in either the PFC (Fig. **5D**) or hippocampus (Fig. **6D**). This suggests that PSD-95 involvement in depression may be region-specific and not strongly associated with these structures — findings consistent with prior studies in human postmortem tissue and in stressed male C57BL/6J mice (Karolewicz et al., 2009; Xue et al., 2023). Similarly, no significant alterations in TrkB (Fig. **5B**, **6B**) or pmTOR/mTOR (Fig. **5C**, **6C**) ratios were detected in both the PFC and hippocampus, although we observed a trend toward restoration of TrkB (Fig. **5B**) and pmTOR/mTOR ratio (Fig. **5C**) in the PFC, which were reduced by chronic stress. This observation aligns with previous reports implicating BDNF-TrkB signaling in the mechanism of action of rapid-acting antidepressants (Duman, 1997; Ghosal et al., 2018; Saarelainen et al., 2003). Given its essential role in neuronal survival and

synaptic plasticity (Drevets et al., 1997; Duman et al., 2000), further research is needed to clarify the contribution of BDNF-TrkB signaling to scopolamine's effects.

5.4 Sustained Antidepressant-Like Effects of Subchronic Coadministration of Scopolamine and the mGlu2 NAM VU6001966 in the UCMS Model

Clinical studies demonstrate that scopolamine exerts rapid and sustained antidepressant effects, although adverse effects limit its therapeutic use (Renner et al., 2005). Preclinical studies suggest that combining scopolamine with metabotropic glutamate receptor ligands may enhance efficacy while mitigating side effects. For example, coadministration of low doses of scopolamine with AMN082, a positive allosteric modulator (PAM) of mGlu7 receptors, or with LY341495, a mGlu2/3 receptor antagonist, has been shown to potentiate scopolamine's antidepressant effects while reducing side effects (Podkowa et al., 2018; Podkowa, Podkowa, et al., 2016). In line with these findings, we first determined which receptor — mGlu2 or mGlu3 — represents the more relevant selective target for antidepressant-like actions. Screening tests revealed that acute administration of the mGlu2 NAM VU6001966, but not the mGlu3 NAM ML289, significantly decreased immobility time in the TST (Fig. **7B–7C**). These results are consistent with other preclinical studies suggesting that the antidepressant-like effects of mGlu2/3 antagonists are primarily mediated by inhibition of mGlu2 rather than mGlu3 receptors. Animal studies have shown that mGlu2 ($Grm2^{-/-}$), but not mGlu3 ($Grm3^{-/-}$), knockout mice display reduced immobility in the FST and exhibit fewer inescapable shock-induced escape failures (Highland et al., 2019). Moreover, deletion of the mGLu2 receptor abolishes the antidepressant-like effects of LY341495, an mGlu2/3 receptor antagonist, whereas this effect is not observed in mGlu3 or mGlu8 knockout mice (Gleason et al., 2013). In addition, both ketamine and its metabolite (2R,6R)-hydroxynorketamine [(2R,6R)-HNK] may exert antidepressant-like effects through mechanisms involving reduced mGlu2 receptor signaling (Zanos et al., 2019). Specifically, ketamine prevented hyperthermia induced by the mGlu2 agonist LY379268, and (2R,6R)-HNK produced similar effects, but only in wild-type (WT) and $Grm3^{-/-}$ knockout mice, not in $Grm2^{-/-}$ mice. Next, we evaluated

the dose-response relationship of the mGlu2 NAM VU6001966 (Fig. 8B–8D) and examined whether VU6001966 could potentiate the antidepressant-like effects of scopolamine in the UCMS model of depression. Our results show that four-day coadministration of subeffective doses of scopolamine and VU6001966 significantly abolished UCMS-induced behavioral effects. Specifically, the combined treatment prevented stress-induced apathy-like state in the splash test (Fig. 9B) and restored sucrose preference (Fig. 9C) in UCMS mice, strongly supporting its rapid antidepressant potential. Importantly, these behavioral parameters were assessed at least 24 hours after the final drug administration — a time window considered to reflect the prolonged effects of rapid-acting antidepressants (Pałucha-Poniewiera, 2018). Similar to scopolamine alone, combined treatment had no behavioral effects in non-stressed control mice, indicating specificity to stress-induced depressive-like states. No significant effects were also observed in the TST (Fig. 9D). There was no significant difference in immobility between naïve and vehicle-treated UCMS mice, although the latter showed a trend toward reduced immobility. This apparent increase in escape-like behavior may reflect elevated anxiety in stressed animals (Anyan & Amir, 2018), lowering baseline threshold in the control group and potentially masking treatment effects. Together, these findings demonstrate that subchronic coadministration of scopolamine and VU6001966 enhances antidepressant efficacy in a stress-dependent manner, while potentially offering a strategy to overcome limitations of scopolamine monotherapy.

5.5 Behavioral Effects of Subchronic Coadministration of Scopolamine and the mGlu2 NAM VU6001966: Role of AMPA and TrkB Receptor Blockade

Converging evidence indicates that activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, along with engagement of the BDNF-TrkB signaling pathway, plays a crucial role in mediating the antidepressant effects of ketamine (Autry et al., 2011; Maeng et al., 2008; W. Zhou et al., 2014). To further investigate their involvement in the antidepressant-like action of scopolamine combined with VU6001966, we examined the behavioral effects of pretreatment with the AMPA receptor antagonist

NBQX and the TrkB receptor antagonist ANA-12 in mice subjected to the UCMS procedure. Pretreatment with NBQX, administered 10 minutes before coadministration of scopolamine with VU6001966, completely abolished their antidepressant-like effects in the TST (Fig. **10D**). Importantly, no changes in locomotor activity that could interfere with the results of the TST were observed (Fig. **12B**). These findings are consistent with earlier reports demonstrating that AMPA receptor activation is required for the behavioral effects of rapid-acting antidepressants. Specifically, NBQX has been reported to block the antidepressant-like effects of ketamine, the mGlu2/3 antagonist MGS0039, as well as scopolamine when coadministered with the mGlu2/3 receptor antagonist LY341495 (Autry et al., 2011; Karasawa et al., 2005; Podkowa, Podkowa, et al., 2016). Interestingly, while NBQX is widely used to block antidepressant-like effects of various compounds, its antidepressant-like effects were observed in the SPT (Fig. **10C**) and TST (Fig. **10D**). This is supported by prior studies suggesting that NBQX's dual action on AMPA and kainate receptors may underlie these paradoxical findings (Libbey et al., 2016). Specifically, while AMPA receptor blockade explains NBQX's ability to antagonize the effects of antidepressants, its simultaneous antagonism of kainate receptors may contribute to its intrinsic antidepressant-like properties (Getachew & Tizabi, 2018). Our behavioral results also demonstrated that pretreatment with the TrkB antagonist ANA-12 completely abolished the sustained antidepressant-like effects of scopolamine-VU6001966 coadministration in both the splash test (Fig. **11B**) and the TST (Fig. **11D**). As with NBQX, locomotor activity was unaffected (Fig. **12C**), confirming that the observed effects were not confounded by nonspecific motor impairments. These findings strongly suggest that TrkB activation is required for the antidepressant-like efficacy of this treatment combination. However, the combination of scopolamine and VU6001966 did not affect sucrose preference in the SPT (Fig. **10C**, **11C**), despite careful replication of experimental conditions. This lack of effect may be explained by a ceiling effect, since vehicle-treated UCMS mice already displayed high sucrose consumption (approximately 80%), potentially masking treatment-related improvements. Consequently, SPT data from these experiments were inconclusive. Due to ethical restrictions imposed by the ethics committee, we were unable to replicate the experiment with an additional stressed cohort.

5.6 Effects of Coadministration of Subeffective Doses of Scopolamine and VU6001966 on TrkB, eEF2, mTOR, and BDNF in the PFC and Hippocampus in the UCMS Model of Depression

To further investigate the role of TrkB — the receptor for BDNF — in the antidepressant-like effects of scopolamine and VU6001966 coadministration, we analyzed the phosphorylated form of TrkB at the Tyr816 site. Our results show that chronic stress induced a significant reduction in TrkB phosphorylation in the PFC (Fig. **13A**), an effect that was fully reversed by the four-day coadministration of scopolamine and VU6001966. Alterations in BDNF-TrkB signaling have been widely implicated in the pathophysiology of mood disorders, with postmortem studies reporting reduced BDNF and TrkB levels in the PFC and hippocampus of patients with major depression or suicide subjects (Dwivedi et al., 2003; Ray et al., 2014; Ray et al., 2011). In contrast, antidepressant treatments increase BDNF levels in both brain and serum, thereby enhancing TrkB-mediated signaling (Chen et al., 2001; Gonul et al., 2005). Interestingly, in our study, the reversal of stress-induced alterations in the pTrkB/TrkB ratio was observed only in the PFC and not in the hippocampus (Fig. **14A**). This regional specificity may suggest a functional dissociation, in which the hippocampus acts as the initiator and the PFC as the executor of sustained antidepressant effects, a mechanism previously proposed for ketamine (Carreno et al., 2016; Jett et al., 2015). Despite these changes in TrkB signaling, BDNF protein levels remained unaltered in both the PFC (Fig. **13D**) and hippocampus (Fig. **14D**). This may reflect methodological limitations in BDNF detection, as tissue collection was performed three days after the final administration — potentially missing transient drug-induced changes. Such transient fluctuations in BDNF could nonetheless trigger downstream adaptations in TrkB signaling, which remain detectable at later time points and may underlie the observed behavioral and molecular effects. In contrast, neither UCMS exposure nor scopolamine-VU6001966 coadministration affected mTOR phosphorylation in the PFC (Fig. **13C**) or hippocampus (Fig. **14C**), suggesting that mTOR does not contribute to the antidepressant-like effects of this drug combination. These findings are consistent with our previous results, in which scopolamine alone also failed to alter

mTOR activation (Fig. **5C**, **6C**). Finally, unlike our earlier findings with scopolamine monotherapy, we observed no effect of either UCMS or the scopolamine-VU6001966 combination on the peEF2/eEF2 ratio (Fig. **13B**, **14B**). This discrepancy may be explained by two factors. First, BDNF — known to promote the active, unphosphorylated form of eEF2 (Hoshi et al., 2018; Takei et al., 2009) — was unchanged (Fig. **13D**, **14D**), which may explain the lack of change in eEF2 phosphorylation. Second, since the peEF2/eEF2 ratio in UCMS-exposed mice was not altered in the present study, there may have been no physiological drive to modulate this pathway, thereby masking potential drug effects.

5.7 Coadministration of Scopolamine and VU6001966: Minimizing Cognitive Adverse Effects

Major depressive disorder is characterized not only by persistent low mood and anhedonia but also by cognitive deficits. While scopolamine has emerged as a promising rapid-acting antidepressant, its anticholinergic properties can negatively affect cognition (Campbell et al., 2009). We therefore evaluated whether coadministration of scopolamine with the mGlu2 NAM VU6001966 mitigates these adverse effects in both naïve and stressed mice. Cognitive function was assessed using the OLT, which measures hippocampus-dependent spatial learning, and the NORT, which assesses non-spatial recognition memory involving multiple brain regions, including the PFC (Denninger et al., 2018). Chronic stress has previously been reported to impair hippocampus-dependent memory in rodent models of depression (Pittenger & Duman, 2008). However, in the present study, no significant memory deficits were observed in vehicle-treated stressed mice compared to controls (Fig. **15**). This is consistent with prior UCMS studies, which indicate that extended exposure (up to 8 weeks) is required to reliably induce cognitive impairments (Alqurashi et al., 2022). We selected a shorter, two-week UCMS procedure due to its suitability for evaluating antidepressant-like behavioral responses. In contrast, longer stress protocols may not be ideal in this context, as animals can develop adaptive changes that reduce their vulnerability to stress (Pałucha-Poniewiera et al., 2020). As a positive control for cognitive impairment, we tested scopolamine at 1 mg/kg, a dose widely used in models of neurodegenerative disorders due to its known cognitive adverse effects (Cieślik et al., 2023; Yadang et al., 2020). As expected, scopolamine at 1 mg/kg significantly impaired

both hippocampus-dependent spatial memory (in the OLT) and recognition memory (in the NORT) (Bird & Burgess, 2008; Denninger et al., 2018). In contrast, coadministration of subeffective doses of scopolamine and VU6001966 did not impair performance in either test. These results suggest that VU6001966 may protect against, or at least fail to exacerbate, the cognitive side effects associated with scopolamine.

Together, these findings are consistent with previous reports highlighting a potential role of mGlu2 antagonists in preserving or improving cognitive function (Higgins et al., 2004), and they support the therapeutic promise of combining scopolamine with VU6001966 to maximize antidepressant efficacy while minimizing cognitive risks.

5.8 Acute Antidepressant-Like Effects of Scopolamine and VU6001966 in Rats

Due to the necessity of conducting further neurochemical studies in rats, resulting from methodological limitations, the next step was to evaluate the antidepressant-like properties of scopolamine and VU6001966 in rats and to establish their dose-response profiles. For this purpose, we employed the forced swim test, a standard paradigm to assess antidepressant activity and to preliminarily assess the involvement of serotonergic and/or noradrenergic mechanisms. In the FST, classical antidepressants such as SSRIs, SNRIs, MAOIs, and TCAs typically reduce immobility time after acute administration (Borsini & Meli, 1988), with increased swimming behavior reflecting serotonergic involvement, while enhanced climbing behavior indicates recruitment of noradrenergic and/or dopaminergic pathways (Detke et al., 1995). Our results demonstrated that the mGlu2 NAM VU6001966 dose-dependently decreased immobility time 45 minutes after intraperitoneal injection (Fig. **17**). Scopolamine also significantly reduced immobility and, in addition, increased both swimming and climbing times (Fig. **16**). Importantly, coadministration of subeffective doses of scopolamine (0.03 mg/kg) and VU6001966 (3 mg/kg) robustly reduced immobility and selectively enhanced climbing behavior (Fig. **18**). The preferential increase in climbing, in the absence of increased swimming, initially suggests that the combined antidepressant-like action of scopolamine and VU6001966 may be mediated primarily through noradrenergic and/or dopaminergic rather than serotonergic mechanisms. These findings are consistent with prior work in mice indicating that the

noradrenergic system plays a key role in the antidepressant-like effects of scopolamine, and that scopolamine can potentiate the efficacy of compounds acting predominantly via noradrenergic modulation (Pałucha-Poniewiera et al., 2017). Importantly, no changes in locomotor activity that could interfere with the results of the FST were observed (Fig. **21**).

5.9 The Effects of Scopolamine and VU6001966 on Extracellular Levels of Serotonin, Dopamine, Glutamate, and GABA in the Rat Frontal Cortex

To further investigate the neurochemical effects of scopolamine and VU6001966, we performed in vivo microdialysis in freely moving rats to monitor extracellular neurotransmitter levels in the frontal cortex (FCX) during the first hours after drug administration. This technique allows the measurement of neurotransmitter fluctuations in the FCX under physiologically relevant conditions by perfusing neurotransmitter-free artificial cerebrospinal fluid at a constant rate and collecting dialysate fractions, thereby reflecting the kinetics of in vivo neurotransmitter release (Di Chiara et al., 1996). Both scopolamine and VU6001966, given alone or in combination, significantly increased 5-HT levels in the FCX (Fig. **19B–19C**), which is consistent with the serotonergic modulation observed after acute administration of conventional antidepressants (Beyer & Cremers, 2008). Although serotonergic and noradrenergic drugs remain first-line treatments for MDD, they often leave patients with residual anhedonia and fail in long-term maintenance therapy (Serretti, 2023). In this context, the dopaminergic effects of the tested compounds are of particular interest. Several studies support the involvement of dopamine dysfunction in depression, particularly in relation to anhedonia and motivational deficits — two core symptoms that are especially hard to cure by SSRIs (Kapur & John Mann, 1992; Yadid & Friedman, 2008). Dopaminergic agents appear to be especially effective in alleviating these symptoms. For example, both preclinical and clinical studies have demonstrated beneficial effects of bupropion, a dopamine reuptake inhibitor, and cariprazine, a partial agonist at dopamine D2 and D3 receptors, in improving anhedonia (Cryan et al., 2003; McIntyre et al., 2025; Papp et al., 2014; Paterson et al., 2007; Tomarken et al., 2004). In-

terestingly, SSRIs with dopaminergic properties, such as sertraline and fluoxetine, as well as the multimodal antidepressant vortioxetine, have also shown greater efficacy in reducing anhedonia compared to SSRIs lacking dopaminergic activity (Serretti, 2023). Our results show that both scopolamine and VU6001966 robustly increased DA levels in the FCX, either alone or in combination (Fig. **19D–19E**). A similar dopaminergic effect has been reported for ketamine, which is strongly associated with anti-anhedonic efficacy (Almeida et al., 2024; Wojtas et al., 2022). The underlying mechanisms of depression appear to be far more complex than the monoaminergic theory suggests, with additional neurochemical systems, particularly the glutamatergic system, playing a crucial role in rapid treatment responses. As the brain's primary and most abundant excitatory neurotransmitter, glutamate is essential for cognition, learning, and mood, domains in which neuroplasticity is critical for adaptation to environmental stressors (Pal, 2021). Our results show that scopolamine also moderately increased extracellular glutamate levels in the FCX (Fig. **20B–20C**), likely due to autoinhibition of mGlu2 autoreceptors by the released glutamate. However, when coadministered with the mGlu2 NAM VU6001966, this inhibition was abolished, as VU6001966 binds to mGlu2 receptors, resulting in a more robust glutamate release. Depressive symptoms have been associated with structural abnormalities and impaired connectivity within cortical and limbic brain regions, driven by dysfunctions in excitatory glutamatergic neurons and inhibitory GABAergic interneurons. According to the neurotrophic hypothesis of depression, reduced neurotrophin levels are linked to increased vulnerability to mood disorders (Duman & Monteggia, 2006). Importantly, acute glutamate release has been shown to upregulate BDNF and nerve growth factor (NGF), thereby supporting synaptic plasticity (Heese et al., 2000; Zafra et al., 1991). In this context, scopolamine and VU6001966 may enhance neuroplastic adaptations by elevating glutamate levels. The effect of scopolamine on glutamate levels in the FCX is consistent with previous studies suggesting that its antidepressant activity is mediated through glutamatergic mechanisms via two pathways: (1) indirect enhancement of pyramidal neuron activity and glutamate transmission in the PFC through blockade of muscarinic stimulatory M1 receptors on GABAergic interneurons, and (2) direct enhancement of pyramidal neuron activity and glutamate transmission in the PFC through blockade of muscarinic inhibitory M2 receptors on glutamatergic neurons (Navarria et al., 2015; Witkin et al., 2014). However, our findings indicate that scopolamine's antidepressant mechanism may

be more complex, also involving activation of the mesocortical pathway and dopamine release in the FCX. Scopolamine administration not only elevated glutamate levels but also increased DA levels in the FCX. Dopamine projections from the medial ventral tegmental area (VTA) appear to be the principal source of DA in the frontal cortex (Zubair et al., 2021). The VTA, a midbrain structure, plays a critical role in motivation and reward processing. Dysfunction of VTA dopaminergic neurons has been linked to neuropsychiatric disorders, including depression and addiction (Polter & Kauer, 2014). Preclinical studies further suggest that increased cholinergic tone in the VTA is associated with pro-depressive and anxiogenic effects (Small et al., 2016), while degeneration of DA neurons in the VTA promotes depressive-like behaviors that can be alleviated by SSRIs or L-DOPA treatment (Winter et al., 2007). Moreover, VTA infusion of an M5 NAM has been shown to attenuate physostigmine-induced anhedonic, anxiogenic, and depressive-like behaviors (Nunes et al., 2020), raising the possibility that scopolamine, as a muscarinic antagonist, may exert similar effects. The antidepressant-like effects of the mGlu2 NAM VU6001966 may also involve modulation of the VTA-PFC pathway. By binding to mGlu2 receptors on glutamatergic neurons within the VTA (Manzoni & Williams, 1999), VU6001966 may enhance glutamate release, which in turn activates dopaminergic neurons and facilitates subsequent DA release in the FCX.

Dopamine can further modulate glutamate and GABA levels in the FCX. Both pyramidal neurons and GABAergic interneurons predominantly express excitatory D1 receptors rather than inhibitory D2 receptors, with pyramidal neurons representing the larger population (Santana et al., 2009). Findings from primate studies demonstrate that the DRD1 receptor is abundantly expressed in glutamatergic pyramidal cells of the PFC (Arnsten et al., 1994). This suggests that dopamine released from mesocortical terminals in the FCX is more likely to stimulate pyramidal cells via D1 receptor activation. Increased D1 receptor signaling may enhance synaptic plasticity by modulating AMPA receptor surface and synaptic expression (Sun et al., 2005). This also aligns with studies using optogenetic stimulation of D1 receptor-expressing pyramidal cells in the mPFC, which found that activation of these neurons produced rapid and long-lasting antidepressant and anxiolytic effects (Hare et al., 2019). Conversely, exposure to chronic stress decreases dopamine levels in the mPFC (Tanaka et al., 2012), while suppression of mesolimbic dopamine transmission increases susceptibility in rodent social defeat models (Chaudhury et al.,

2013; Shinohara et al., 2017). Sustained activation of the VTA-PFC circuit gradually enhances the excitability of parvalbumin-positive (PV+) interneurons in the PFC, leading to GABA release (Zhong et al., 2020). Conversely, the proposed mechanism of scopolamine's action involves inhibition of GABAergic interneurons, leading to a reduction in GABA release. This opposing balance may account for the absence of significant changes in extracellular GABA levels following administration of the tested compounds, with only a subtle increase observed when scopolamine and VU6001966 are coadministered (Fig. **20D**–**20E**).

5.10 Coadministration of Scopolamine and VU6001966 — Proposed Antidepressant-Like Mechanism of Action in a Nutshell

Neuronal functions are dynamic processes that occur in response to various stimuli. Negative stimuli, such as chronic stress, are known to induce both depressive symptoms and alterations in neural plasticity (Dean & Keshavan, 2017; Duman et al., 2000). Based on current knowledge and our findings, we propose that synaptic reorganization in the PFC is a key event in the rapid antidepressant-like effects of scopolamine and VU6001966. Our neurochemical studies suggest that VU6001966 may enhance scopolamine-induced glutamate release in the frontal cortex. Scopolamine may act by blocking M1 muscarinic receptors on GABAergic neurons, thereby reducing GABA release and disinhibiting glutamatergic neurons, which results in excessive glutamate release. In addition, scopolamine may block M2 receptors located on glutamatergic terminals, further promoting glutamate release. Similarly, VU6001966 exerts its effects by antagonizing mGlu2 receptors at glutamatergic nerve endings, leading to disinhibition and enhanced glutamate release. The increased glutamate likely activates AMPA receptors, which in turn stimulate the release of BDNF. BDNF then binds to and activates TrkB receptors via autophosphorylation, initiating downstream signaling cascades that promote neuronal survival, growth, and synaptic plasticity — processes implicated in the therapeutic actions of antidepressants. Dopamine released from mesocortical terminals in the FCX activates pyramidal cells through D1 receptors, thereby promoting synaptic plasticity by modulating AMPA

receptor expression. Additionally, scopolamine-induced dephosphorylation of eEF2 in the PFC is thought to relieve suppression of BDNF translation, thereby promoting BDNF protein synthesis.

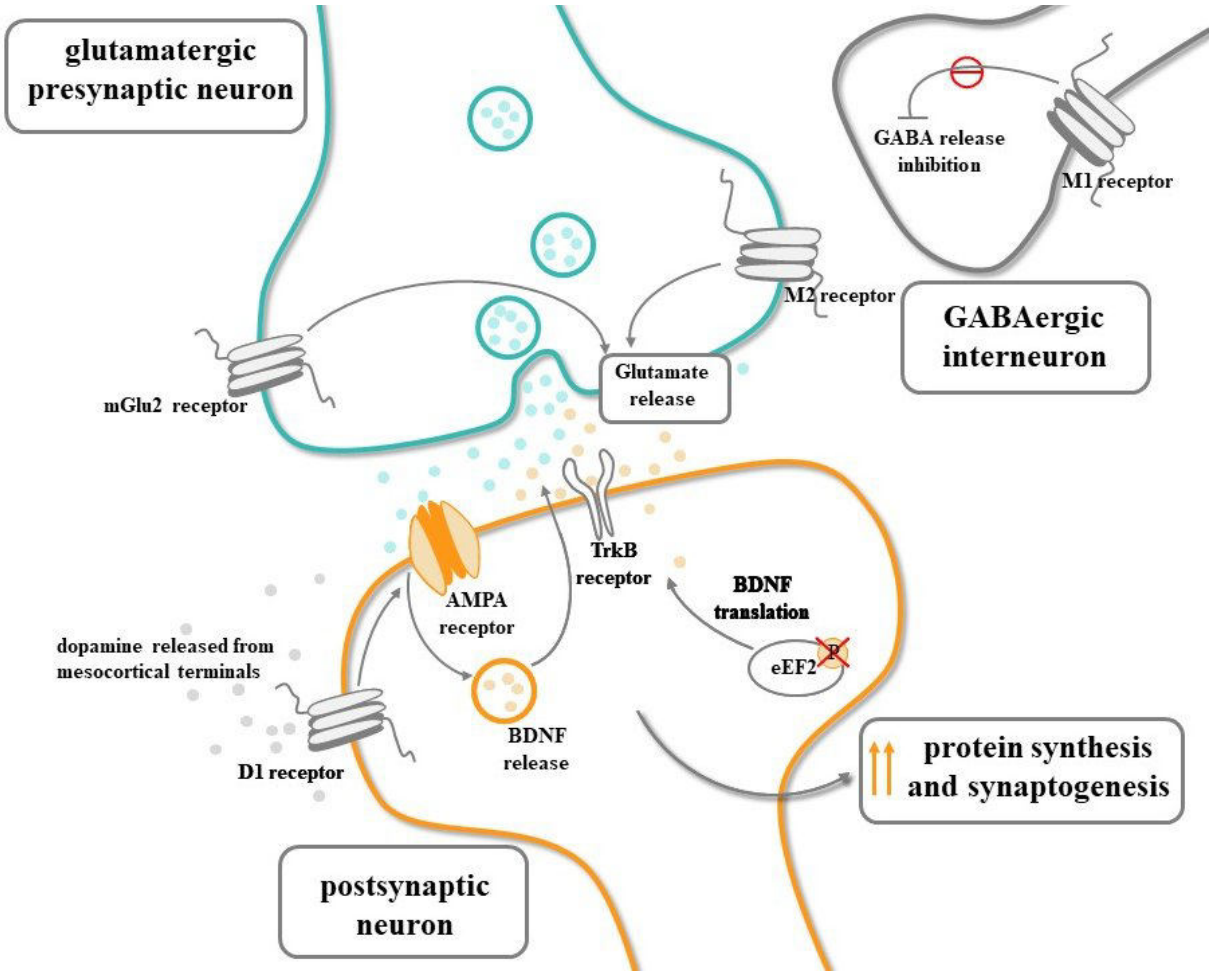


Figure 22: Proposed mechanism of action for combined administration of scopolamine with the mGlu2 NAM VU6001966. Scopolamine blocks M1 muscarinic receptors on GABAergic neurons, reducing GABA release and thereby disinhibiting glutamatergic neurons, which results in glutamate overflow. In addition, blockade of M2 receptors on glutamatergic terminals further increases glutamate release. VU6001966 enhances this effect by antagonizing mGlu2 receptors at glutamatergic nerve endings, leading to additional disinhibition of glutamate release. The excess glutamate activates AMPA receptors, which in turn triggers BDNF release and subsequent activation of TrkB receptors. Additional mechanisms have also been proposed, including scopolamine-induced dephosphorylation of eEF2, which promotes BDNF protein synthesis, and activation of D1 receptors by dopamine released from mesocortical terminals, which facilitates synaptic plasticity via modulation of AMPA receptor expression. Together, these mechanisms lead to enhanced protein synthesis and synaptogenesis.

5.11 Limitations and Future Directions

Our findings open the door to future investigations on the duration and sustainability of the antidepressant-like behavioral effects observed with combined scopolamine and VU6001966 administration. Based on our results, these effects persist for at least two days after the last combined treatment and three days following scopolamine treatment alone (as demonstrated by the additional FST test on day 3). Future experiments should assess whether these effects extend over longer periods. Given the presumed involvement of signaling pathways regulating synaptic plasticity, it would also be valuable to examine the impact of chronic stress and scopolamine-VU6001966 coadministration on dendritic spine morphology and density. Moreover, all experiments were conducted in male rodents, limiting the generalizability of our findings. Potential sex- and species-specific differences highlight the need for replication in female animals (Furey et al., 2010) and careful consideration when extrapolating to human depression. Another limitation is that our neurochemical analyses were restricted to acute drug effects in naïve rats. Future studies should incorporate animal models with a depressive-like phenotype, which more closely reflect the core symptoms of major depression, including behavioral despair and anhedonia (Petković & Chaudhury, 2022). The latter is particularly important considering our data suggesting potential drug effects on the VTA. In addition, because scopolamine is a nonselective muscarinic receptor antagonist, assessing acetylcholine levels is crucial. However, this was not possible due to technical issues encountered during microdialysis. Addressing this limitation in future studies will be crucial. Furthermore, exploring how the drug combination modulates neurotransmitter release in other brain regions (e.g., hippocampus, VTA) may provide important mechanistic insights. While our study focused on scopolamine with an mGlu2 NAM VU6001966, other combinations of scopolamine should also be considered. To date, there have been a few clinical attempts using scopolamine augmentation. Scopolamine combined with SSRI escitalopram did not improve depressive symptoms in MDD (J. Zhou et al., 2020), whereas the combination of scopolamine and naltrexone showed promising results (Taub, 2019). Similarly, preclinical data on ketamine and scopolamine coadministration support further translational efforts (Martin et al., 2017). Expanding such comparative studies may help to identify the most effective and safe augmentation strategies.

Chapter 6

Conclusion

In summary, our results support the notion that augmenting scopolamine with the mGlu2 NAM VU6001966 not only enhances its antidepressant-like efficacy but may also reduce the risk of cognitive adverse effects. This strategy may have important clinical implications, as it could allow for lower therapeutic doses of scopolamine while preserving cognitive function. Further research is needed to clarify the underlying mechanisms and establish translational potential.

Bibliography

Abdallah, C. G., Jackowski, A., Salas, R., Gupta, S., Sato, J. R., Mao, X., Coplan, J. D., Shungu, D. C., & Mathew, S. J. (2017). The nucleus accumbens and ketamine treatment in major depressive disorder. *Neuropsychopharmacology*, *42*(8), 1739–1746. https://doi.org/10.1038/npp.2017.49

Abdallah, C. G., Sanacora, G., Duman, R. S., & Krystal, J. H. (2018). The neurobiology of depression, ketamine and rapid-acting antidepressants: Is it glutamate inhibition or activation? *Pharmacology & Therapeutics*, *190*, 148–158. https://doi.org/10.1016/j.pharmthera.2018.05.010

Aleem, D., & Tohid, H. (2018). Pro-inflammatory cytokines, biomarkers, genetics and the immune system: A mechanistic approach of depression and psoriasis. *Revista Colombiana de Psiquiatría (English Ed.)*, *47*(3), 177–186. https://doi.org/10.1016/j.rcpeng.2018.05.002

Almeida, T. M., Generoso, I. P., Rosa, D. A. A., Pinheiro, T. B., Foletto, L. D., Jorge, G. M. T., Grilo, L. B., da Silva, U. R. L., Cordeiro, Q., & Uchida, R. R. (2024). The anti-anhedonic effects of ketamine in the treatment of resistant unipolar and bipolar depression: A systematic review and meta-analysis of current data. *Journal of Affective Disorders Reports*, *17*, 100829. https://doi.org/10.1016/j.jadr.2024.100829

Alqurashi, G. K., Hindi, E. A., Zayed, M. A., Abd El-Aziz, G. S., Alturkistani, H. A., Ibrahim, R. F., Al-thepyani, M. A., Bakhlgi, R., Alzahrani, N. A., Ashraf, G. M., & Alghamdi, B. S. (2022). The impact of chronic unpredictable mild stress-induced depression on spatial, recognition and reference memory tasks in mice: Behavioral and histological study. *Behavioral Sciences*, *12*(6), 166. https://doi.org/10.3390/bs12060166

Altinyazar, V., Sirin, F. B., Sutcu, R., Eren, I., & Omurlu, I. K. (2016). The red blood cell acetylcholinesterase levels of depressive patients with suicidal behavior in an agricultural area. *Indian Journal of Clinical Biochemistry*, *31*(4), 473–479. https://doi.org/10.1007/s12291-016-0558-9

Anderson, I. M. (1998). Ssris versus tricyclic antidepressants in depressed inpatients: A meta-analysis of efficacy and tolerability. *Depression and Anxiety*, *7 Suppl 1*, 11–17.

Anyan, J., & Amir, S. (2018). Too depressed to swim or too afraid to stop? a reinterpretation of the forced swim test as a measure of anxiety-like behavior. *Neuropsychopharmacology*, *43*(5), 931–933. https://doi.org/10.1038/npp.2017.260

Arnone, D., McIntosh, A. M., Ebmeier, K. P., Munafò, M. R., & Anderson, I. M. (2012). Magnetic resonance imaging studies in unipolar depression: Systematic review and meta-regression analyses. *European Neuropsychopharmacology*, *22*(1), 1–16. https://doi.org/10.1016/j.euroneuro.2011.05.003

Arnsten, A. F., Cai, J. X., Murphy, B. L., & Goldman-Rakic, P. S. (1994). Dopamine d1 receptor mechanisms in the cognitive performance of young adult and aged monkeys. *Psychopharmacology*, *116*(2), 143–151. https://doi.org/10.1007/BF02245056

Autry, A. E., Adachi, M., Nosyreva, E., Na, E. S., Los, M. F., Cheng, P., Kavalali, E. T., & Monteggia, L. M. (2011). Nmda receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature*, *475*(7354), 91–95. https://doi.org/10.1038/nature10130

Bagley, J., & Moghaddam, B. (1997). Temporal dynamics of glutamate efflux in the prefrontal cortex and in the hippocampus following repeated stress: Effects of pretreatment with saline or diazepam. *Neuroscience*, *77*(1), 65–73. https://doi.org/10.1016/S0306-4522(96)00435-6

Ballard, E. D., Yarrington, J. S., Farmer, C. A., Lener, M. S., Kadriu, B., Lally, N., Williams, D., Machado-Vieira, R., Niciu, M. J., Park, L., & Zarate, C. A. (2018). Parsing the heterogeneity of depression: An exploratory factor analysis across commonly used depression rating scales. *Journal of Affective Disorders*, *231*, 51–57. https://doi.org/10.1016/j.jad.2018.01.027

Ballard, M. E., Basso, A. M., Gallagher, K. B., Browman, K. E., Fox, G. B., Drescher, K. U., Gross, G., Decker, M. W., Rueter, L. E., & Zhang, M. (2007). The drug-

induced helplessness test: An animal assay for assessing behavioral despair in response to neuroleptic treatment. *Psychopharmacology*, *190*(1), 1–11. https://doi.org/10.1007/s00213-006-0577-y

Belleau, E. L., Treadway, M. T., & Pizzagalli, D. A. (2019). The impact of stress and major depressive disorder on hippocampal and medial prefrontal cortex morphology. *Biological Psychiatry*, *85*(6), 443–453. https://doi.org/10.1016/j.biopsych.2018.09.031

Bellone, C., Lüscher, C., & Mameli, M. (2008). Mechanisms of synaptic depression triggered by metabotropic glutamate receptors. *Cellular and Molecular Life Sciences*, *65*(18), 2913–2923. https://doi.org/10.1007/s00018-008-8263-3

Berman, R. M., Cappiello, A., Anand, A., Oren, D. A., Heninger, G. R., Charney, D. S., & Krystal, J. H. (2000). Antidepressant effects of ketamine in depressed patients. *Biological Psychiatry*, *47*(4), 351–354. https://doi.org/10.1016/S0006-3223(99)00230-9

Beyer, C. E., & Cremers, T. I. F. H. (2008). Do selective serotonin reuptake inhibitors acutely increase frontal cortex levels of serotonin? *European Journal of Pharmacology*, *580*(3), 350–354. https://doi.org/10.1016/j.ejphar.2007.11.028

Bird, C. M., & Burgess, N. (2008). The hippocampus and memory: Insights from spatial processing. *Nature Reviews Neuroscience*, *9*(3), 182–194. https://doi.org/10.1038/nrn2335

Birmaher, B., Dahl, R. E., Williamson, D. E., Perel, J. M., Brent, D. A., Axelson, D. A., Kaufman, J., Dorn, L. D., Stull, S., Rao, U., & Ryan, N. D. (2000). Growth hormone secretion in children and adolescents at high risk for major depressive disorder. *Archives of General Psychiatry*, *57*(9), 867. https://doi.org/10.1001/archpsyc.57.9.867

Borsini, F., & Meli, A. (1988). Is the forced swimming test a suitable model for revealing antidepressant activity? *Psychopharmacology*, *94*(2). https://doi.org/10.1007/BF00176837

Bosker, F. J., Tanke, M. A. C., Jongsma, M. E., Cremers, T. I. F. H., Jagtman, E., Pietersen, C. Y., van der Hart, M. G. C., Gladkevich, A. V., Kema, I. P., & Westerink, B. H. C. (2010). Biochemical and behavioral effects of long-term citalopram administration and discontinuation in rats: Role of serotonin synthesis. *Neurochemistry International*, *57*(8), 948–957. https://doi.org/10.1016/j.neuint.2010.10.001

Buckley, T. M., & Schatzberg, A. F. (2010). A pilot study of the phase angle between cortisol and melatonin in major depression – a potential biomarker? *Journal of Psychiatric Research*, *44*(2), 69–74. https://doi.org/10.1016/j.jpsychires.2009.06.012

Campbell, N., Boustani, M., Limbil, T., Ott, C., Fox, C., Maidment, I., Schubert, C. C., Munger, S., Fick, D., Miller, D., & Gulati, R. (2009). The cognitive impact of anticholinergics: A clinical review. *Clinical Interventions in Aging*, *4*, 225–233. https://doi.org/10.2147/cia.s5358

Carreno, F. R., Donegan, J. J., Boley, A. M., Shah, A., DeGuzman, M., Frazer, A., & Lodge, D. J. (2016). Activation of a ventral hippocampus–medial prefrontal cortex pathway is both necessary and sufficient for an antidepressant response to ketamine. *Molecular Psychiatry*, *21*(9), 1298–1308. https://doi.org/10.1038/mp.2015.176

Casey, D. E. (1979). Mood alterations during deanol therapy. *Psychopharmacology*, *62*(2), 187–191. https://doi.org/10.1007/BF00427135

Chaki, S., Yoshikawa, R., Hirota, S., Shimazaki, T., Maeda, M., Kawashima, N., Yoshimizu, T., Yasuhara, A., Sakagami, K., Okuyama, S., Nakanishi, S., & Nakazato, A. (2004). Mgs0039: A potent and selective group ii metabotropic glutamate receptor antagonist with antidepressant-like activity. *Neuropharmacology*, *46*(4), 457–467. https://doi.org/10.1016/j.neuropharm.2003.10.009

Chau, D. T., Rada, P., Kosloff, R. A., Taylor, J. L., & Hoebel, B. G. (2001). Nucleus accumbens muscarinic receptors in the control of behavioral depression: Antidepressant-like effects of local m1 antagonist in the porsolt swim test. *Neuroscience*, *104*(3), 791–798. https://doi.org/10.1016/S0306-4522(01)00133-6

Chaudhury, D., et al. (2013). Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons. *Nature*, *493*, 532–536. https://doi.org/10.1038/nature11713

Chen, B., Dowlatshahi, D., MacQueen, G. M., Wang, J.-F., & Young, L. T. (2001). Increased hippocampal bdnf immunoreactivity in subjects treated with antidepressant medication. *Biological Psychiatry*, *50*(4), 260–265. https://doi.org/10.1016/S0006-3223(01)01083-6

Choi, D. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron*, *1*(8), 623–634. https://doi.org/10.1016/0896-6273(88)90162-6

Cieślik, P., Borska, M., & Wierońska, J. M. (2023). A comparative study of the impact of no-related agents on mk-801- or scopolamine-induced cognitive impairments in the morris water maze. *Brain Sciences*, *13*(3). https://doi.org/10.3390/brainsci13030410

Conn, P. J., & Pin, J. P. (1997). Pharmacology and functions of metabotropic glutamate receptors. *Annual Review of Pharmacology and Toxicology*, *37*, 205–237. https://doi.org/10.1146/annurev.pharmtox.37.1.205

Cryan, J. F., Bruijnzeel, A. W., Skjei, K. L., & Markou, A. (2003). Bupropion enhances brain reward function and reverses the affective and somatic aspects of nicotine withdrawal in the rat. *Psychopharmacology*, *168*(3), 347–358. https://doi.org/10.1007/s00213-003-1445-7

Cui, L., Li, S., Wang, S., Wu, X., Liu, Y., Yu, W., Wang, Y., Tang, Y., Xia, M., & Li, B. (2024). Major depressive disorder: Hypothesis, mechanism, prevention and treatment. *Signal Transduction and Targeted Therapy*, *9*(1), 30. https://doi.org/10.1038/s41392-024-01738-y

Davis, K., Hollander, E., Davidson, M., Davis, B., Mohs, R., & Horvath, T. (1987). Induction of depression with oxotremorine in patients with alzheimer's disease. *American Journal of Psychiatry*, *144*(4), 468–471. https://doi.org/10.1176/ajp.144.4.468

Davis, K. L., Berger, P. A., Hollister, L. E., & Defraites, E. (1978). Physostigmine in mania. *Archives of General Psychiatry*, *35*(1), 119. https://doi.org/10.1001/archpsyc.1978.01770250121012

Dean, J., & Keshavan, M. (2017). The neurobiology of depression: An integrated view. *Asian Journal of Psychiatry*, *27*, 101–111. https://doi.org/10.1016/j.ajp.2017.01.025

Delgado, P. L., Price, L. H., Miller, H. L., Salomon, R. M., Licinio, J., Krystal, J. H., Heninger, G. R., & Charney, D. S. (1991). Rapid serotonin depletion as a provocative challenge test for patients with major depression: Relevance to antidepressant action and the neurobiology of depression. *Psychopharmacology Bulletin*, *27*(3), 321–330.

Denninger, J. K., Smith, B. M., & Kirby, E. D. (2018). Novel object recognition and object location behavioral testing in mice on a budget. *Journal of Visualized Experiments*, (141). https://doi.org/10.3791/58593

Detke, M. J., Rickels, M., & Lucki, I. (1995). Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology*, *121*(1), 66–72. https://doi.org/10.1007/BF02245592

Di Chiara, G., Tanda, G., & Carboni, E. (1996). Estimation of in-vivo neurotransmitter release by brain microdialysis: The issue of validity. *Behavioural Pharmacology*, *7*(7), 640–657.

Dong, C., Tian, Z., Fujita, Y., Fujita, A., Hino, N., Iijima, M., & Hashimoto, K. (2022). Antidepressant-like actions of the mglu2/3 receptor antagonist tp0178894 in the chronic social defeat stress model: Comparison with escitalopram. *Pharmacology Biochemistry and Behavior*, *212*, 173316. https://doi.org/10.1016/j.pbb.2021.173316

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E. K., & Lanctôt, K. L. (2010). A meta-analysis of cytokines in major depression. *Biological Psychiatry*, *67*(5), 446–457. https://doi.org/10.1016/j.biopsych.2009.09.033

Drevets, W. C., Price, J. L., Simpson, J. R., Todd, R. D., Reich, T., Vannier, M., & Raichle, M. E. (1997). Subgenual prefrontal cortex abnormalities in mood disorders. *Nature*, *386*(6627), 824–827. https://doi.org/10.1038/386824a0

Dulawa, S. C., & Janowsky, D. S. (2019). Cholinergic regulation of mood: From basic and clinical studies to emerging therapeutics. *Molecular Psychiatry*, *24*(5), 694–709. https://doi.org/10.1038/s41380-018-0219-x

Duman, R. S. (1997). A molecular and cellular theory of depression. *Archives of General Psychiatry*, *54*(7), 597. https://doi.org/10.1001/archpsyc.1997.01830190015002

Duman, R. S., Malberg, J., Nakagawa, S., & D'Sa, C. (2000). Neuronal plasticity and survival in mood disorders. *Biological Psychiatry*, *48*(8), 732–739. https://doi.org/10.1016/S0006-3223(00)00935-5

Duman, R. S., & Monteggia, L. M. (2006). A neurotrophic model for stress-related mood disorders. *Biological Psychiatry*, *59*(12), 1116–1127. https://doi.org/10.1016/j.biopsych.2006.02.013

Dwivedi, Y., Rizavi, H. S., Conley, R. R., Roberts, R. C., Tamminga, C. A., & Pandey, G. N. (2003). Altered gene expression of brain-derived neurotrophic factor and receptor tyrosine kinase b in postmortem brain of suicide subjects. *Archives of General Psychiatry*, *60*(8), 804. https://doi.org/10.1001/archpsyc.60.8.804

Ellis, J. S., Zarate, C. A., Luckenbaugh, D. A., & Furey, M. L. (2014). Antidepressant treatment history as a predictor of response to scopolamine: Clinical implications. *Journal of Affective Disorders*, *162*, 39–42. https://doi.org/10.1016/j.jad.2014.03.010

Fava, G. A. (2020). May antidepressant drugs worsen the conditions they are supposed to treat? the clinical foundations of the oppositional model of tolerance. *Therapeutic Advances in Psychopharmacology*, *10*. https://doi.org/10.1177/2045125320970325

Feyissa, A. M., Woolverton, W. L., Miguel-Hidalgo, J. J., Wang, Z., Kyle, P. B., Hasler, G., Stockmeier, C. A., Iyo, A. H., & Karolewicz, B. (2010). Elevated level of metabotropic glutamate receptor 2/3 in the prefrontal cortex in major depression. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *34*(2), 279–283. https://doi.org/10.1016/j.pnpbp.2009.11.018

Furey, M. L., & Drevets, W. C. (2006). Antidepressant efficacy of the antimuscarinic drug scopolamine. *Archives of General Psychiatry*, *63*(10), 1121. https://doi.org/10.1001/archpsyc.63.10.1121

Furey, M. L., Khanna, A., Hoffman, E. M., & Drevets, W. C. (2010). Scopolamine produces larger antidepressant and antianxiety effects in women than in men. *Neuropsychopharmacology*, *35*(12), 2479–2488. https://doi.org/10.1038/npp.2010.131

Gaynes, B. N., Lux, L., Gartlehner, G., Asher, G., Forman-Hoffman, V., Green, J., Boland, E., Weber, R. P., Randolph, C., Bann, C., Coker-Schwimmer, E., Viswanathan, M., & Lohr, K. N. (2020). Defining treatment-resistant depression. *Depression and Anxiety*, *37*(2), 134–145. https://doi.org/10.1002/da.22968

Gershon, S., & Shaw, F. H. (1961). Psychiatric sequelæ of chronic exposure to organophosphorus insecticides. *The Lancet*, *277*(7191), 1371–1374. https://doi.org/10.1016/S0140-6736(61)92004-9

Getachew, B., & Tizabi, Y. (2018). Both ketamine and nbqx attenuate alcohol-withdrawal induced depression in male rats. *Journal of Drug and Alcohol Research*, *08*(01). https://doi.org/10.4303/jdar/236069

Ghosal, S., Bang, E., Yue, W., Hare, B. D., Lepack, A. E., Girgenti, M. J., & Duman, R. S. (2018). Activity-dependent brain-derived neurotrophic factor release is required for the rapid antidepressant actions of scopolamine. *Biological Psychiatry*, *83*(1), 29–37. https://doi.org/10.1016/j.biopsych.2017.06.017

Gleason, S. D., Li, X., Smith, I. A., Ephlin, J. D., Wang, X.-S., Heinz, B. A., Carter, J. H., Baez, M., Yu, J., Bender, D. M., & Witkin, J. M. (2013). Mglu2/3 agonist-induced hyperthermia: An in vivo assay for detection of mglu2/3 receptor antagonism and its relation to antidepressant-like efficacy in mice. *CNS & Neurological Disorders - Drug Targets*, *12*(5), 554–566. https://doi.org/10.2174/18715273113129990079

Gonul, A. S., Akdeniz, F., Taneli, F., Donat, O., Eker, Ç., & Vahip, S. (2005). Effect of treatment on serum brain–derived neurotrophic factor levels in depressed patients. *European Archives of Psychiatry and Clinical Neuroscience*, *255*(6), 381–386. https://doi.org/10.1007/s00406-005-0578-6

Gosrani, S. P., Jester, H. M., Zhou, X., Ryazanov, A. G., & Ma, T. (2020). Repression of eef2 kinase improves deficits in novel object recognition memory in aged mice. *Neurobiology of Aging*, *95*, 154–160. https://doi.org/10.1016/j.neurobiolaging.2020.07.016

Goto, A. (2022). Synaptic plasticity during systems memory consolidation. *Neuroscience Research*, *183*, 1–6. https://doi.org/10.1016/j.neures.2022.05.008

Hannestad, J. O., Cosgrove, K. P., DellaGioia, N. F., Perkins, E., Bois, F., Bhagwagar, Z., Seibyl, J. P., McClure-Begley, T. D., Picciotto, M. R., & Esterlis, I. (2013). Changes in the cholinergic system between bipolar depression and euthymia as measured with [123i]5ia single photon emission computed tomography. *Biological Psychiatry*, *74*(10), 768–776. https://doi.org/10.1016/j.biopsych.2013.04.004

Heese, K., Otten, U., Mathivet, P., Raiteri, M., Marescaux, C., & Bernasconi, R. (2000). Gabab receptor antagonists elevate both mrna and protein levels of the neurotrophins nerve growth factor (ngf) and brain-derived neurotrophic factor (bdnf) but not neurotrophin-3 (nt-3) in brain and spinal cord of rats. *Neuropharmacology*, *39*(3), 449–462. https://doi.org/10.1016/S0028-3908(99)00166-5

Higgins, G. A., Ballard, T. M., Kew, J. N. C., Richards, J. G., Kemp, J. A., Adam, G., Woltering, T., Nakanishi, S., & Mutel, V. (2004). Pharmacological manipulation

of mglu2 receptors influences cognitive performance in the rodent. *Neuropharmacology*, *46*(7), 907–917. https://doi.org/10.1016/j.neuropharm.2004.01.018

Hoshi, O., Sugizaki, A., Cho, Y., & Takei, N. (2018). Bdnf reduces eef2 phosphorylation and enhances novel protein synthesis in the growth cones of dorsal root ganglia neurons. *Neurochemical Research*, *43*(6), 1242–1249. https://doi.org/10.1007/s11064-018-2541-8

Howren, M. B., Lamkin, D. M., & Suls, J. (2009). Associations of depression with c-reactive protein, il-1, and il-6: A meta-analysis. *Psychosomatic Medicine*, *71*(2), 171–186. https://doi.org/10.1097/PSY.0b013e3181907c1b

Hrabovska, A., Duysen, E. G., Sanders, J. D., Murrin, L. C., & Lockridge, O. (2005). Delivery of human acetylcholinesterase by adeno-associated virus to the acetylcholinesterase knockout mouse. *Chemico-Biological Interactions*, *157-158*, 71–78. https://doi.org/10.1016/j.cbi.2005.10.014

Huang, T., Balasubramanian, R., Yao, Y., Clish, C. B., Shadyab, A. H., Liu, B., Tworoger, S. S., Rexrode, K. M., Manson, J. E., Kubzansky, L. D., & Hankinson, S. E. (2021). Associations of depression status with plasma levels of candidate lipid and amino acid metabolites: A meta-analysis of individual data from three independent samples of us postmenopausal women. *Molecular Psychiatry*, *26*(7), 3315–3327. https://doi.org/10.1038/s41380-020-00870-9

Jaffe, D. H., Rive, B., & Denee, T. R. (2019). The humanistic and economic burden of treatment-resistant depression in europe: A cross-sectional study. *BMC Psychiatry*, *19*(1), 247. https://doi.org/10.1186/s12888-019-2222-4

Janowsky, D. S., Davis, J. M., El-Yousef, M. K., & Sekerke, H. J. (1972). A cholinergic-adrenergic hypothesis of mania and depression. *The Lancet*, *300*(7778), 632–635. https://doi.org/10.1016/S0140-6736(72)93021-8

Janowsky, D. S., el-Yousef, K., Davis, J. M., & Sekerke, H. J. (1973). Parasympathetic suppression of manic symptoms by physostigmine. *Archives of General Psychiatry*, *28*(4), 542. https://doi.org/10.1001/archpsyc.1973.01750340072012

Janowsky, D. S., Risch, S. C., & Gillin, J. C. (1983). Adrenergic-cholinergic balance and the treatment of affective disorders. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *7*(2-3), 297–307. https://doi.org/10.1016/0278-5846(83)90119-7

Jett, J. D., Boley, A. M., Girotti, M., Shah, A., Lodge, D. J., & Morilak, D. A. (2015). Antidepressant-like cognitive and behavioral effects of acute ketamine administration associated with plasticity in the ventral hippocampus to medial prefrontal cortex pathway. *Psychopharmacology*, *232*(17), 3123–3133. https://doi.org/10.1007/s00213-015-3957-3

Joffe, M. E., Santiago, C. I., Oliver, K. H., Maksymetz, J., Harris, N. A., Engers, J. L., Lindsley, C. W., Winder, D. G., & Conn, P. J. (2020). Mglu2 and mglu3 negative allosteric modulators divergently enhance thalamocortical transmission and exert rapid antidepressant-like effects. *Neuron*, *105*(1), 46–59.e3. https://doi.org/10.1016/j.neuron.2019.09.044

Kapur, S., & John Mann, J. (1992). Role of the dopaminergic system in depression. *Biological Psychiatry*, *32*(1), 1–17. https://doi.org/10.1016/0006-3223(92)90137-O

Karasawa, J., Shimazaki, T., Kawashima, N., & Chaki, S. (2005). Ampa receptor stimulation mediates the antidepressant-like effect of a group ii metabotropic glutamate receptor antagonist. *Brain Research*, *1042*(1), 92–98. https://doi.org/10.1016/j.brainres.2005.02.032

Karolewicz, B., Szebeni, K., Gilmore, T., Maciag, D., Stockmeier, C. A., & Ordway, G. A. (2009). Elevated levels of nr2a and psd-95 in the lateral amygdala in depression. *The International Journal of Neuropsychopharmacology*, *12*(2), 143. https://doi.org/10.1017/S1461145708008985

Katz, R. J., & Hersh, S. (1981). Amitriptyline and scopolamine in an animal model of depression. *Neuroscience & Biobehavioral Reviews*, *5*(2), 265–271. https://doi.org/10.1016/0149-7634(81)90008-7

Kempton, M. J., Salvador, Z., Munafò, M. R., Geddes, J. R., Simmons, A., Frangou, S., & Williams, S. C. R. (2011). Structural neuroimaging studies in major depressive disorder. *Archives of General Psychiatry*, *68*(7), 675. https://doi.org/10.1001/archgenpsychiatry.2011.60

Kendler, K. S. (2020). The origin of our modern concept of depression—the history of melancholia from 1780-1880. *JAMA Psychiatry*, *77*(8), 863. https://doi.org/10.1001/jamapsychiatry.2019.4709

Kim, T., Xu, C., & Amsterdam, J. D. (2019). Relative effectiveness of tricyclic antidepressant versus monoamine oxidase inhibitor monotherapy for treatment-resistant depression. *Journal of Affective Disorders*, *250*, 199–203. https://doi.org/10.1016/j.jad.2019.03.028

Krystal, J. H., Kavalali, E. T., & Monteggia, L. M. (2024). Ketamine and rapid antidepressant action: New treatments and novel synaptic signaling mechanisms. *Neuropsychopharmacology*, *49*(1), 41–50. https://doi.org/10.1038/s41386-023-01629-w

Kuhn, R. (1958). The treatment of depressive states with g 22355 (imipramine hydrochloride). *American Journal of Psychiatry*, *115*(5), 459–464. https://doi.org/10.1176/ajp.115.5.459

Lane, R., Baldwin, D., & Preskorn, S. (1995). The ssris: Advantages, disadvantages and differences. *Journal of Psychopharmacology*, *9*(2), 163–178. https://doi.org/10.1177/0269881195009002011

Lapin, I. P., & Oxenkrug, G. F. (1969). Intensification of the central serotoninergic processes as a possible determinant of the thymoleptic effect. *The Lancet*, *293*(7586), 132–136. https://doi.org/10.1016/S0140-6736(69)91140-4

Larrea, A., Sánchez-Sánchez, L., Diez-Martin, E., Elexpe, A., Torrecilla, M., Astigarraga, E., & Barreda-Gómez, G. (2024). Mitochondrial metabolism in major depressive disorder: From early diagnosis to emerging treatment options. *Journal of Clinical Medicine*, *13*(6), 1727. https://doi.org/10.3390/jcm13061727

Li, N., Lee, B., Liu, R.-J., Banasr, M., Dwyer, J. M., Iwata, M., Li, X.-Y., Aghajanian, G., & Duman, R. S. (2010). Mtor-dependent synapse formation underlies the rapid antidepressant effects of nmda antagonists. *Science*, *329*(5994), 959–964. https://doi.org/10.1126/science.1190287

Li, S.-X., Han, Y., Xu, L.-Z., Yuan, K., Zhang, R.-X., Sun, C.-Y., Xu, D.-F., Yuan, M., Deng, J.-H., Meng, S.-Q., Gao, X.-J., Wen, Q., Liu, L.-J., Zhu, W.-L., Xue, Y.-X., Zhao, M., Shi, J., & Lu, L. (2018). Uncoupling dapk1 from nmda receptor glun2b subunit exerts rapid antidepressant-like effects. *Molecular Psychiatry*, *23*(3), 597–608. https://doi.org/10.1038/mp.2017.85

Libbey, J. E., Hanak, T. J., Doty, D. J., Wilcox, K. S., & Fujinami, R. S. (2016). Nbqx, a highly selective competitive antagonist of ampa and ka ionotropic glutamate recep-

tors, increases seizures and mortality following picornavirus infection. *Experimental Neurology*, *280*, 89–96. https://doi.org/10.1016/j.expneurol.2016.04.010

Liu, Q., He, H., Yang, J., Feng, X., Zhao, F., & Lyu, J. (2020). Changes in the global burden of depression from 1990 to 2017: Findings from the global burden of disease study. *Journal of Psychiatric Research*, *126*, 134–140. https://doi.org/10.1016/j.jpsychires.2019.08.002

Loomer, H. P., Saunders, J. C., & Kline, N. S. (1957). A clinical and pharmacodynamic evaluation of iproniazid as a psychic energizer. *Psychiatric Research Reports*, *8*, 129–141.

Machado-Vieira, R., Baumann, J., Wheeler-Castillo, C., Latov, D., Henter, I. D., Salvadore, G., & Zarate, C. A. (2010). The timing of antidepressant effects: A comparison of diverse pharmacological and somatic treatments. *Pharmaceuticals*, *3*(1), 19–41. https://doi.org/10.3390/ph3010019

Maeng, S., Zarate, C. A., Du, J., Schloesser, R. J., McCammon, J., Chen, G., & Manji, H. K. (2008). Cellular mechanisms underlying the antidepressant effects of ketamine: Role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. *Biological Psychiatry*, *63*(4), 349–352. https://doi.org/10.1016/j.biopsych.2007.05.028

Manzoni, O. J., & Williams, J. T. (1999). Presynaptic regulation of glutamate release in the ventral tegmental area during morphine withdrawal. *The Journal of Neuroscience*, *19*(15), 6629–6636. https://doi.org/10.1523/JNEUROSCI.19-15-06629.1999

Markov, D. D., & Novosadova, E. V. (2022). Chronic unpredictable mild stress model of depression: Possible sources of poor reproducibility and latent variables. *Biology*, *11*(11), 1621. https://doi.org/10.3390/biology11111621

Martin, A. E., Schober, D. A., Nikolayev, A., Tolstikov, V. V., Anderson, W. H., Higgs, R. E., Kuo, M.-S., Laksmanan, A., Catlow, J. T., Li, X., Felder, C. C., & Witkin, J. M. (2017). Further evaluation of mechanisms associated with the antidepressantlike signature of scopolamine in mice. *CNS & Neurological Disorders - Drug Targets*, *16*(4). https://doi.org/10.2174/1871527316666170309142646

McEwen, B. S., Nasca, C., & Gray, J. D. (2016). Stress effects on neuronal structure: Hippocampus, amygdala, and prefrontal cortex. *Neuropsychopharmacology*, *41*(1), 3–23. https://doi.org/10.1038/npp.2015.171

McIntyre, R. S., Maletic, V., Masand, P., Wilson, A. C., Yu, J., Adams, J. L., & Kerolous, M. (2025). The effect of adjunctive cariprazine on symptoms of anhedonia in patients with major depressive disorder. *Journal of Affective Disorders*, *385*, 119366. https://doi.org/10.1016/j.jad.2025.05.026

Mineur, Y. S., Obayemi, A., Wigestrand, M. B., Fote, G. M., Calarco, C. A., Li, A. M., & Picciotto, M. R. (2013). Cholinergic signaling in the hippocampus regulates social stress resilience and anxiety- and depression-like behavior. *Proceedings of the National Academy of Sciences*, *110*(9), 3573–3578. https://doi.org/10.1073/pnas.1219731110

Moćko, P., Śladowska, K., Kawalec, P., Babii, Y., & Pilc, A. (2023). The potential of scopolamine as an antidepressant in major depressive disorder: A systematic review of randomized controlled trials. *Biomedicines*, *11*(10), 2636. https://doi.org/10.3390/biomedicines11102636

Modestin, J., Hunger, J., & Schwartz, R. B. (1973). Über die depressogene wirkung von physostigmin. *Archiv Fur Psychiatrie Und Nervenkrankheiten*, *218*(1), 67–77. https://doi.org/10.1007/BF00347089

Moncrieff, J., Cooper, R. E., Stockmann, T., Amendola, S., Hengartner, M. P., & Horowitz, M. A. (2023). The serotonin theory of depression: A systematic umbrella review of the evidence. *Molecular Psychiatry*, *28*(8), 3243–3256. https://doi.org/10.1038/s41380-022-01661-0

Monroe, S. M., & Harkness, K. L. (2022). Major depression and its recurrences: Life course matters. *Annual Review of Clinical Psychology*, *18*(1), 329–357. https://doi.org/10.1146/annurev-clinpsy-072220-021440

Moriguchi, S., Takamiya, A., Noda, Y., Horita, N., Wada, M., Tsugawa, S., Plitman, E., Sano, Y., Tarumi, R., ElSalhy, M., Katayama, N., Ogyu, K., Miyazaki, T., Kishimoto, T., Graff-Guerrero, A., Meyer, J. H., Blumberger, D. M., Daskalakis, Z. J., Mimura, M., & Nakajima, S. (2019). Glutamatergic neurometabolite levels in major depressive disorder: A systematic review and meta-analysis of proton

magnetic resonance spectroscopy studies. *Molecular Psychiatry*, *24*(7), 952–964. https://doi.org/10.1038/s41380-018-0252-9

Muly, E. C., Mania, I., Guo, J., & Rainnie, D. G. (2007). Group ii metabotropic glutamate receptors in anxiety circuitry: Correspondence of physiological response and subcellular distribution. *Journal of Comparative Neurology*, *505*(6), 682–700. https://doi.org/10.1002/cne.21525

Murack, M., Chandrasegaram, R., Smith, K. B., Ah-Yen, E. G., Rheaume, É., Malette-Guyon, É., Nanji, Z., Semchishen, S. N., Latus, O., Messier, C., & Ismail, N. (2021). Chronic sleep disruption induces depression-like behavior in adolescent male and female mice and sensitization of the hypothalamic-pituitary-adrenal axis in adolescent female mice. *Behavioural Brain Research*, *399*, 113001. https://doi.org/10.1016/j.bbr.2020.113001

Musazzi, L., Treccani, G., & Popoli, M. (2015). Functional and structural remodeling of glutamate synapses in prefrontal and frontal cortex induced by behavioral stress. *Frontiers in Psychiatry*, *6*. https://doi.org/10.3389/fpsyt.2015.00060

Navarria, A., Wohleb, E. S., Voleti, B., Ota, K. T., Dutheil, S., Lepack, A. E., Dwyer, J. M., Fuchikami, M., Becker, A., Drago, F., & Duman, R. S. (2015). Rapid antidepressant actions of scopolamine: Role of medial prefrontal cortex and m1-subtype muscarinic acetylcholine receptors. *Neurobiology of Disease*, *82*, 254–261. https://doi.org/10.1016/j.nbd.2015.06.012

Nibuya, M., Morinobu, S., & Duman, R. (1995). Regulation of bdnf and trkb mrna in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *The Journal of Neuroscience*, *15*(11), 7539–7547. https://doi.org/10.1523/JNEUROSCI.15-11-07539.1995

Niswender, C. M., & Conn, P. J. (2010). Metabotropic glutamate receptors: Physiology, pharmacology, and disease. *Annual Review of Pharmacology and Toxicology*, *50*(1), 295–322. https://doi.org/10.1146/annurev.pharmtox.011008.145533

Nunes, E. J., Rupprecht, L. E., Foster, D. J., Lindsley, C. W., Conn, P. J., & Addy, N. A. (2020). Examining the role of muscarinic m5 receptors in vta cholinergic modulation of depressive-like and anxiety-related behaviors in rats. *Neuropharmacology*, *171*, 108089. https://doi.org/10.1016/j.neuropharm.2020.108089

Nurnberger, J. I., Jimerson, D. C., Simmons-Alling, S., Tamminga, C., Nadi, N. S., Lawrence, D., Sitaram, N., Gillin, J. C., & Gershon, E. S. (1983). Behavioral, physiological, and neuroendocrine responses to arecoline in normal twins and "well state" bipolar patients. *Psychiatry Research*, *9*(3), 191–200. https://doi.org/10.1016/0165-1781(83)90043-4

Olds, M. E., & Domino, E. F. (1969). Comparison of muscarinic and nicotinic cholinergic agonists on self-stimulation behavior. *The Journal of Pharmacology and Experimental Therapeutics*, *166*(2), 189–204.

Öngür, D., Drevets, W. C., & Price, J. L. (1998). Glial reduction in the subgenual prefrontal cortex in mood disorders. *Proceedings of the National Academy of Sciences*, *95*(22), 13290–13295. https://doi.org/10.1073/pnas.95.22.13290

Overstreet, D. H., Friedman, E., Mathé, A. A., & Yadid, G. (2005). The flinders sensitive line rat: A selectively bred putative animal model of depression. *Neuroscience & Biobehavioral Reviews*, *29*(4–5), 739–759. https://doi.org/10.1016/j.neubiorev.2005.03.015

Pal, M. M. (2021). Glutamate: The master neurotransmitter and its implications in chronic stress and mood disorders. *Frontiers in Human Neuroscience*, *15*. https://doi.org/10.3389/fnhum.2021.722323

Pałucha-Poniewiera, A., Brański, P., Lenda, T., & Pilc, A. (2010). The antidepressant-like action of metabotropic glutamate 7 receptor agonist n,n'-bis(diphenylmethyl)-1,2-ethanediamine (amn082) is serotonin-dependent. *Journal of Pharmacology and Experimental Therapeutics*, *334*(3), 1066–1074. https://doi.org/10.1124/jpet.110.169730

Pałucha-Poniewiera, A., Podkowa, K., Lenda, T., & Pilc, A. (2017). The involvement of monoaminergic neurotransmission in the antidepressant-like action of scopolamine in the tail suspension test. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *79*, 155–161. https://doi.org/10.1016/j.pnpbp.2017.06.022

Pałucha-Poniewiera, A., Podkowa, K., & Pilc, A. (2019). Role of ampa receptor stimulation and trkb signaling in the antidepressant-like effect of ketamine co-administered with a group ii mglu receptor antagonist, ly341495, in the forced swim test in rats. *Behavioural Pharmacology*, *30*(6), 471–477. https://doi.org/10.1097/FBP.0000000000000471

Pałucha-Poniewiera, A., Podkowa, K., & Rafało-Ulińska, A. (2021). The group ii mglu receptor antagonist ly341495 induces a rapid antidepressant-like effect and enhances the effect of ketamine in the chronic unpredictable mild stress model of depression in c57bl/6j mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *109*, 110239. https://doi.org/10.1016/j.pnpbp.2020.110239

Pałucha-Poniewiera, A., Podkowa, K., Rafało-Ulińska, A., Brański, P., & Burnat, G. (2020). The influence of the duration of chronic unpredictable mild stress on the behavioural responses of c57bl/6j mice. *Behavioural Pharmacology*, *31*(6), 574–582. https://doi.org/10.1097/FBP.0000000000000564

Pałucha-Poniewiera, A., Wierońska, J. M., Brański, P., Stachowicz, K., Chaki, S., & Pilc, A. (2010). On the mechanism of the antidepressant-like action of group ii mglu receptor antagonist, mgs0039. *Psychopharmacology*, *212*(4), 523–535. https://doi.org/10.1007/s00213-010-1978-5

Papp, M., Gruca, P., Lasoń-Tyburkiewicz, M., Adham, N., Kiss, B., & Gyertyán, I. (2014). Attenuation of anhedonia by cariprazine in the chronic mild stress model of depression. *Behavioural Pharmacology*, *25*(5 and 6), 567–574. https://doi.org/10.1097/FBP.0000000000000070

Park, L., Furey, M., Nugent, A. C., Farmer, C., Ellis, J., Szczepanik, J., Lener, M. S., & Zarate, C. A. (2019). Neurophysiological changes associated with antidepressant response to ketamine not observed in a negative trial of scopolamine in major depressive disorder. *International Journal of Neuropsychopharmacology*, *22*(1), 10–18. https://doi.org/10.1093/ijnp/pyy051

Parker, G., Roy, K., Wilhelm, K., & Mitchell, P. (2001). Assessing the comparative effectiveness of antidepressant therapies. *The Journal of Clinical Psychiatry*, *62*(2), 117–125. https://doi.org/10.4088/JCP.v62n0209

Paterson, N. E., Balfour, D. J., & Markou, A. (2007). Chronic bupropion attenuated the anhedonic component of nicotine withdrawal in rats via inhibition of dopamine reuptake in the nucleus accumbens shell. *European Journal of Neuroscience*, *25*(10), 3099–3108. https://doi.org/10.1111/j.1460-9568.2007.05546.x

Paxinos, G., & Watson, C. (1998). *The rat brain in stereotaxic coordinates*. Academic Press.

Paykel, E. S. (2008). Basic concepts of depression. *Dialogues in Clinical Neuroscience*, *10*(3), 279–289. https://doi.org/10.31887/DCNS.2008.10.3/espaykel

Pech, J., Forman, J., Kessing, L. V., & Knorr, U. (2018). Poor evidence for putative abnormalities in cerebrospinal fluid neurotransmitters in patients with depression versus healthy non-psychiatric individuals: A systematic review and meta-analyses of 23 studies. *Journal of Affective Disorders*, *240*, 6–16. https://doi.org/10.1016/j.jad.2018.07.031

Petković, A., & Chaudhury, D. (2022). Encore: Behavioural animal models of stress, depression and mood disorders. *Frontiers in Behavioral Neuroscience*, *16*. https://doi.org/10.3389/fnbeh.2022.931964

Pittenger, C., & Duman, R. S. (2008). Stress, depression, and neuroplasticity: A convergence of mechanisms. *Neuropsychopharmacology*, *33*(1), 88–109. https://doi.org/10.1038/sj.npp.1301574

Podkowa, K., Pilc, A., Podkowa, A., Sałat, K., Marciniak, M., & Pałucha-Poniewiera, A. (2018). The potential antidepressant action and adverse effects profile of scopolamine co-administered with the mglu7 receptor allosteric agonist amn082 in mice. *Neuropharmacology*, *141*, 214–222. https://doi.org/10.1016/j.neuropharm.2018.08.022

Podkowa, K., Pochwat, B., Brański, P., Pilc, A., & Pałucha-Poniewiera, A. (2016). Group ii mglu receptor antagonist ly341495 enhances the antidepressant-like effects of ketamine in the forced swim test in rats. *Psychopharmacology*, *233*(15–16), 2901–2914. https://doi.org/10.1007/s00213-016-4325-7

Podkowa, K., Podkowa, A., Sałat, K., Lenda, T., Pilc, A., & Pałucha-Poniewiera, A. (2016). Antidepressant-like effects of scopolamine in mice are enhanced by the group ii mglu receptor antagonist ly341495. *Neuropharmacology*, *111*, 169–179. https://doi.org/10.1016/j.neuropharm.2016.08.031

Polter, A. M., & Kauer, J. A. (2014). Stress and vta synapses: Implications for addiction and depression. *European Journal of Neuroscience*, *39*(7), 1179–1188. https://doi.org/10.1111/ejn.12490

Porsolt, R. D., Anton, G., Blavet, N., & Jalfre, M. (1978). Behavioural despair in rats: A new model sensitive to antidepressant treatments. *European Journal of Pharmacology*, *47*(4), 379–391. https://doi.org/10.1016/0014-2999(78)90118-8

Porsolt, R. D., Le Pichon, M., & Jalfre, M. (1977). Depression: A new animal model sensitive to antidepressant treatments. *Nature*, *266*(5604), 730–732. https://doi.org/10.1038/266730a0

Rafało-Ulińska, A., Brański, P., & Pałucha-Poniewiera, A. (2022). Combined administration of (r)-ketamine and the mglu2/3 receptor antagonist ly341495 induces rapid and sustained effects in the cums model of depression via a trkb/bdnf-dependent mechanism. *Pharmaceuticals*, *15*(2), 125. https://doi.org/10.3390/ph15020125

Rathbun, R. C., & Slater, I. H. (1963). Amitriptyline and nortriptyline as antagonists of central and peripheral cholinergic activation. *Psychopharmacologia*, *4*(2), 114–125. https://doi.org/10.1007/BF00413329

Ray, M. T., Shannon Weickert, C., & Webster, M. J. (2014). Decreased bdnf and trkb mrna expression in multiple cortical areas of patients with schizophrenia and mood disorders. *Translational Psychiatry*, *4*(5), e389–e389. https://doi.org/10.1038/tp.2014.26

Ray, M. T., Weickert, C. S., Wyatt, E., & Webster, M. J. (2011). Decreased bdnf, trkb-tk+ and gad 67 mrna expression in the hippocampus of individuals with schizophrenia and mood disorders. *Journal of Psychiatry and Neuroscience*, *36*(3), 195–203. https://doi.org/10.1503/jpn.100048

Remick, R. A. (1988). Anticholinergic side effects of tricyclic antidepressants and their management. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *12*(2–3), 225–231. https://doi.org/10.1016/0278-5846(88)90039-5

Renner, U. D., Oertel, R., & Kirch, W. (2005). Pharmacokinetics and pharmacodynamics in clinical use of scopolamine. *Therapeutic Drug Monitoring*, *27*(5), 655–665. https://doi.org/10.1097/01.ftd.0000168293.48226.57

Reynolds, E. H., & Wilson, J. V. K. (2013). Depression and anxiety in babylon. *Journal of the Royal Society of Medicine*, *106*(12), 478–481. https://doi.org/10.1177/0141076813486262

Reznikov, L. R., Grillo, C. A., Piroli, G. G., Pasumarthi, R. K., Reagan, L. P., & Fadel, J. (2007). Acute stress-mediated increases in extracellular glutamate levels in the rat amygdala: Differential effects of antidepressant treatment. *European Journal of Neuroscience*, *25*(10), 3109–3114. https://doi.org/10.1111/j.1460-9568.2007.05560.x

Richelson, E. (1994). Pharmacology of antidepressants - characteristics of the ideal drug. *Mayo Clinic Proceedings*, *69*(11), 1069–1081. https://doi.org/10.1016/S0025-6196(12)61375-5

Risch, S. C., Cohen, R. M., Janowsky, D. S., Kalin, N. H., Sitaram, N., Christian Gillin, J., & Murphy, D. L. (1981). Physostigmine induction of depressive symptomatology in normal human subjects. *Psychiatry Research*, *4*(1), 89–94. https://doi.org/10.1016/0165-1781(81)90012-3

Rowntree, D. W., Nevin, S., & Wilson, A. (1950). The effects of diisopropylfluorophosphonate in schizophrenia and manic depressive psychosis. *Journal of Neurology, Neurosurgery & Psychiatry*, *13*(1), 47–62. https://doi.org/10.1136/jnnp.13.1.47

Ryazanov, A. G., Shestakova, E. A., & Natapov, P. G. (1988). Phosphorylation of elongation factor 2 by ef-2 kinase affects rate of translation. *Nature*, *334*(6178), 170–173. https://doi.org/10.1038/334170a0

Saarelainen, T., Hendolin, P., Lucas, G., Koponen, E., Sairanen, M., MacDonald, E., Agerman, K., Haapasalo, A., Nawa, H., Aloyz, R., Ernfors, P., & Castrén, E. (2003). Activation of the trkb neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. *The Journal of Neuroscience*, *23*(1), 349–357. https://doi.org/10.1523/JNEUROSCI.23-01-00349.2003

Sanacora, G., & Banasr, M. (2013). From pathophysiology to novel antidepressant drugs: Glial contributions to the pathology and treatment of mood disorders. *Biological Psychiatry*, *73*(12), 1172–1179. https://doi.org/10.1016/j.biopsych.2013.03.032

Sanacora, G., Treccani, G., & Popoli, M. (2012). Towards a glutamate hypothesis of depression. *Neuropharmacology*, *62*(1), 63–77. https://doi.org/10.1016/j.neuropharm.2011.07.036

Santana, N., Mengod, G., & Artigas, F. (2009). Quantitative analysis of the expression of dopamine d1 and d2 receptors in pyramidal and gabaergic neurons of the rat prefrontal cortex. *Cerebral Cortex*, *19*(4), 849–860. https://doi.org/10.1093/cercor/bhn134

Saricicek, A., Esterlis, I., Maloney, K. H., Mineur, Y. S., Ruf, B. M., Muralidharan, A., Chen, J. I., Cosgrove, K. P., Kerestes, R., Ghose, S., Tamminga, C. A., Pittman, B., Bois, F., Tamagnan, G., Seibyl, J., Picciotto, M. R., Staley, J. K., & Bhagwagar,

Z. (2012). Persistent β2 *-nicotinic acetylcholinergic receptor dysfunction in major depressive disorder. *American Journal of Psychiatry*, *169*(8), 851–859. https://doi.org/10.1176/appi.ajp.2012.11101546

Schildkraut, J. J. (1965). The catecholamine hypothesis of affective disorders: A review of supporting evidence. *American Journal of Psychiatry*, *122*(5), 509–522. https://doi.org/10.1176/ajp.122.5.509

Selikoff, I. J., & Robitzek, E. H. (1952). Tuberculosis chemotherapy with hydrazine derivatives of isonicotinic acid. *Diseases of the Chest*, *21*(4), 385–438. https://doi.org/10.1378/chest.21.4.385

Serretti, A. (2023). Anhedonia and depressive disorders. *Clinical Psychopharmacology and Neuroscience*, *21*(3), 401–409. https://doi.org/10.9758/cpn.23.1086

Shi, X., Zhou, X., Chen, G., Luo, W., Zhou, C., He, T., Naik, M. T., Jiang, Q., Marshall, J., & Cao, C. (2024). Targeting the postsynaptic scaffolding protein psd-95 enhances bdnf signaling to mitigate depression-like behaviors in mice. *Science Signaling*, *17*(834). https://doi.org/10.1126/scisignal.adn4556

Shinohara, R., et al. (2017). Dopamine d1 receptor subtype mediates acute stress-induced dendritic growth in excitatory neurons of the medial prefrontal cortex and contributes to suppression of stress susceptibility in mice. *Molecular Psychiatry*, *23*, 1717–1730. https://doi.org/10.1038/mp.2017.137

Small, K. M., Nunes, E., Hughley, S., & Addy, N. A. (2016). Ventral tegmental area muscarinic receptors modulate depression and anxiety-related behaviors in rats. *Neuroscience Letters*, *616*, 80–85. https://doi.org/10.1016/j.neulet.2016.01.057

Steru, L., Chermat, R., Thierry, B., & Simon, P. (1985). The tail suspension test: A new method for screening antidepressants in mice. *Psychopharmacology*, *85*(3), 367–370. https://doi.org/10.1007/BF00428203

Strekalova, T., & Steinbusch, H. W. M. (2010). Measuring behavior in mice with chronic stress depression paradigm. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *34*(2), 348–361. https://doi.org/10.1016/j.pnpbp.2009.12.014

Sun, X., Sun, C., Zhai, L., & Dong, W. (2019). A selective m1 and m3 receptor antagonist, penehyclidine hydrochloride, exerts antidepressant-like effect in mice. *Neurochemical Research*, *44*(12), 2723–2732. https://doi.org/10.1007/s11064-019-02891-5

Sun, X., Zhao, Y., & Wolf, M. E. (2005). Dopamine receptor stimulation modulates ampa receptor synaptic insertion in prefrontal cortex neurons. *Journal of Neuroscience*, *25*(32), 7342–7351. https://doi.org/10.1523/JNEUROSCI.4603-04.2005

Taha, E., Gildish, I., Gal-Ben-Ari, S., & Rosenblum, K. (2013). The role of eef2 pathway in learning and synaptic plasticity. *Neurobiology of Learning and Memory*, *105*, 100–106. https://doi.org/10.1016/j.nlm.2013.04.015

Takei, N., Kawamura, M., Ishizuka, Y., Kakiya, N., Inamura, N., Namba, H., & Nawa, H. (2009). Brain-derived neurotrophic factor enhances the basal rate of protein synthesis by increasing active eukaryotic elongation factor 2 levels and promoting translation elongation in cortical neurons. *Journal of Biological Chemistry*, *284*(39), 26340–26348. https://doi.org/10.1074/jbc.M109.023010

Tamminga, C., Smith, R. C., Chang, S., Haraszti, J. S., & Davis, J. M. (1976). Depression associated with oral choline. *The Lancet*, *308*(7991), 905. https://doi.org/10.1016/S0140-6736(76)90562-6

Tanaka, K., Furuyashiki, T., Kitaoka, S., Senzai, Y., Imoto, Y., Segi-Nishida, E., Deguchi, Y., Breyer, R. M., Breyer, M. D., & Narumiya, S. (2012). Prostaglandin e2-mediated attenuation of mesocortical dopaminergic pathway is critical for susceptibility to repeated social defeat stress in mice. *Journal of Neuroscience*, *32*(12), 4319–4329. https://doi.org/10.1523/JNEUROSCI.5952-11.2012

Taub, N. (2019). Naltrexone and scopolamine rapidly reduce symptoms of major depressive disorder (mdd): A double blinded randomized controlled pilot study. *Open Journal of Depression*, *08*(01), 1–4. https://doi.org/10.4236/ojd.2019.81001

Toide, K. (1989). Effects of scopolamine on extracellular acetylcholine and choline levels and on spontaneous motor activity in freely moving rats measured by brain dialysis. *Pharmacology Biochemistry and Behavior*, *33*(1), 109–113. https://doi.org/10.1016/0091-3057(89)90438-3

Tomarken, A. J., Dichter, G. S., Freid, C., Addington, S., & Shelton, R. C. (2004). Assessing the effects of bupropion sr on mood dimensions of depression. *Journal of Affective Disorders*, *78*(3), 235–241. https://doi.org/10.1016/S0165-0327(02)00306-3

Tran, I., & Gellner, A.-K. (2023). Long-term effects of chronic stress models in adult mice. *Journal of Neural Transmission*, *130*(9), 1133–1151. https://doi.org/10.1007/s00702-023-02598-6

Tytgat, G. N. (2007). Hyoscine butylbromide: A review of its use in the treatment of abdominal cramping and pain. *Drugs*, *67*(9), 1343–1357. https://doi.org/10.2165/00003495-200767090-00007

Van Praag, H. M., Korf, J., & Puite, J. (1970). 5-hydroxyindoleacetic acid levels in the cerebrospinal fluid of depressive patients treated with probenecid. *Nature*, *225*(5239), 1259–1260. https://doi.org/10.1038/2251259b0

van Enkhuizen, J., Milienne-Petiot, M., Geyer, M. A., & Young, J. W. (2015). Modeling bipolar disorder in mice by increasing acetylcholine or dopamine: Chronic lithium treats most, but not all features. *Psychopharmacology*, *232*(18), 3455–3467. https://doi.org/10.1007/s00213-015-4000-4

Venzala, E., García-García, A. L., Elizalde, N., & Tordera, R. M. (2013). Social vs. environmental stress models of depression from a behavioural and neurochemical approach. *European Neuropsychopharmacology*, *23*(7), 697–708. https://doi.org/10.1016/j.euroneuro.2012.05.010

Videbech, P., & Ravnkilde, B. (2004). Hippocampal volume and depression: A meta-analysis of mri studies. *American Journal of Psychiatry*, *161*(11), 1957–1966. https://doi.org/10.1176/appi.ajp.161.11.1957

Voleti, B., Navarria, A., Liu, R.-J., Banasr, M., Li, N., Terwilliger, R., Sanacora, G., Eid, T., Aghajanian, G., & Duman, R. S. (2013). Scopolamine rapidly increases mammalian target of rapamycin complex 1 signaling, synaptogenesis, and antidepressant behavioral responses. *Biological Psychiatry*, *74*(10), 742–749. https://doi.org/10.1016/j.biopsych.2013.04.025

Wierońska, J. M., & Pilc, A. (2019). Depression and schizophrenia viewed from the perspective of amino acidergic neurotransmission: Antipodes of psychiatric disorders. *Pharmacology & Therapeutics*, *193*, 75–82. https://doi.org/10.1016/j.pharmthera.2018.08.010

Willner, P. (2017). The chronic mild stress (cms) model of depression: History, evaluation and usage. *Neurobiology of Stress*, *6*, 78–93. https://doi.org/10.1016/j.ynstr.2016.08.002

Winter, C., von Rumohr, A., Mundt, A., Petrus, D., Klein, J., Lee, T., Morgenstern, R., Kupsch, A., & Juckel, G. (2007). Lesions of dopaminergic neurons in the substantia nigra pars compacta and in the ventral tegmental area enhance depressive-like

behavior in rats. *Behavioural Brain Research*, *184*(2), 133–141. https://doi.org/10.1016/j.bbr.2007.07.002

Witkin, J. M., Overshiner, C., Li, X., Catlow, J. T., Wishart, G. N., Schober, D. A., Heinz, B. A., Nikolayev, A., Tolstikov, V. V., Anderson, W. H., Higgs, R. E., Kuo, M.-S., & Felder, C. C. (2014). M1 and m2 muscarinic receptor subtypes regulate antidepressant-like effects of the rapidly acting antidepressant scopolamine. *The Journal of Pharmacology and Experimental Therapeutics*, *351*(2), 448–456. https://doi.org/10.1124/jpet.114.216804

Wojtas, A., Bysiek, A., Wawrzczak-Bargiela, A., Szych, Z., Majcher-Maślanka, I., Herian, M., Maćkowiak, M., & Gołembiowska, K. (2022). Effect of psilocybin and ketamine on brain neurotransmitters, glutamate receptors, dna and rat behavior. *International Journal of Molecular Sciences*, *23*(12), 6713. https://doi.org/10.3390/ijms23126713

World Health Organization. (2011). *Global burden of mental disorders and the need for a comprehensive, coordinated response from health and social sectors at the country level–report by the secretariat.*

World Health Organization. (2023). Depressive disorder [Accessed: 2025-09-08]. https://www.who.int/news-room/fact-sheets/detail/depression

Wozniak, K. M., Rojas, C., Wu, Y. W., & Slusher, B. S. (2012). The role of glutamate signaling in pain processes and its regulation by gcp ii inhibition. *Current Medicinal Chemistry*, *19*(9), 1323–1334. https://doi.org/10.2174/092986712799462630

Xue, S.-G., He, J.-G., Lu, L.-L., Song, S.-J., Chen, M.-M., Wang, F., & Chen, J.-G. (2023). Enhanced tarp-γ8-psd-95 coupling in excitatory neurons contributes to the rapid antidepressant-like action of ketamine in male mice. *Nature Communications*, *14*(1), 7971. https://doi.org/10.1038/s41467-023-42780-8

Yadang, F. S. A., Nguezeye, Y., Kom, C. W., Betote, P. H. D., Mamat, A., Tchokouaha, L. R. Y., Taiwé, G. S., Agbor, G. A., & Bum, E. N. (2020). Scopolamine-induced memory impairment in mice: Neuroprotective effects of carissa edulis (forssk.) valh (apocynaceae) aqueous extract. *International Journal of Alzheimer's Disease*, 1–10. https://doi.org/10.1155/2020/6372059

Yadid, G., & Friedman, A. (2008). Dynamics of the dopaminergic system as a key component to the understanding of depression. In *Serotonin–dopamine interaction:*

Experimental evidence and therapeutic relevance (pp. 265–286). Elsevier. https://doi.org/10.1016/S0079-6123(08)00913-8

Yu, H., Lv, D., Shen, M., Zhang, Y., Zhou, D., Chen, Z., & Wang, C. (2019). Bdnf mediates the protective effects of scopolamine in reserpine-induced depression-like behaviors via up-regulation of 5-htt and tph1. *Psychiatry Research*, *271*, 328–334. https://doi.org/10.1016/j.psychres.2018.12.015

Zafra, F., Castrén, E., Thoenen, H., & Lindholm, D. (1991). Interplay between glutamate and gamma-aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons. *Proceedings of the National Academy of Sciences*, *88*(22), 10037–10041. https://doi.org/10.1073/pnas.88.22.10037

Zanos, P., Highland, J. N., Stewart, B. W., Georgiou, P., Jenne, C. E., Lovett, J., Morris, P. J., Thomas, C. J., Moaddel, R., Zarate, C. A., & Gould, T. D. (2019). 2r,6r-hydroxynorketamine exerts mglu2 receptor-dependent antidepressant actions. *Proceedings of the National Academy of Sciences*, *116*(13), 6441–6450. https://doi.org/10.1073/pnas.1819540116

Zarate, C. A., Singh, J. B., Carlson, P. J., Brutsche, N. E., Ameli, R., Luckenbaugh, D. A., Charney, D. S., & Manji, H. K. (2006). A randomized trial of an n-methyl-d-aspartate antagonist in treatment-resistant major depression. *Archives of General Psychiatry*, *63*(8), 856. https://doi.org/10.1001/archpsyc.63.8.856

Zhong, P., Qin, L., & Yan, Z. (2020). Dopamine differentially regulates response dynamics of prefrontal cortical principal neurons and interneurons to optogenetic stimulation of inputs from ventral tegmental area. *Cerebral Cortex*, *30*(8), 4402–4409. https://doi.org/10.1093/cercor/bhaa027

Zhou, J., Yang, J., Zhu, X., Zghoul, T., Feng, L., Chen, R., & Wang, G. (2020). The effects of intramuscular administration of scopolamine augmentation in moderate to severe major depressive disorder: A randomized, double-blind, placebo-controlled trial. *Therapeutic Advances in Psychopharmacology*, *10*. https://doi.org/10.1177/2045125320938556

Zhou, W., Wang, N., Yang, C., Li, X.-M., Zhou, Z.-Q., & Yang, J.-J. (2014). Ketamine-induced antidepressant effects are associated with ampa receptors-mediated up-

regulation of mtor and bdnf in rat hippocampus and prefrontal cortex. *European Psychiatry*, *29*(7), 419–423. https://doi.org/10.1016/j.eurpsy.2013.10.005

Zubair, M., Murris, S. R., Isa, K., Onoe, H., Koshimizu, Y., Kobayashi, K., Vanduffel, W., & Isa, T. (2021). Divergent whole brain projections from the ventral midbrain in macaques. *Cerebral Cortex*, *31*(6), 2913–2931. https://doi.org/10.1093/cercor/bhaa399