Levetiracetam prevents neurophysiological changes and preserves cognitive function in the HIV-1 TAT transgenic mouse model of HIV-associated neurocognitive disorder

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Dedication

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Abstract

Worldwide, 39 million people are currently HIV-positive; around half of these individuals also suffer from HIV-associated neurocognitive disorder (HAND). HAND symptoms range from subclinical cognitive impairment to HIV-associated dementia. Once people living with HIV (PLWH) have one form of HAND, they are at much higher risk of developing more severe types, which are correlated with worse prognosis and decreased survival. Currently, there is no treatment available specifically for HAND and its mechanism of manifestation has not been fully elucidated.

Combined antiretroviral therapy (cART), the first line treatment for HIV, has greatly extended the lifespan of PLWH. However, cART has not improved the prevalence of HAND. This suggests that HIV can continue to cause damage even at virologically controlled levels. Indeed, the virus lies latent in microglial viral reservoirs and continues to produce HIV-associated proteins, several of which have been implicated in HAND. There is conclusive evidence that trans-activator of transcription (TAT) persists in the central nervous system despite cART and contributes to the development of HAND.

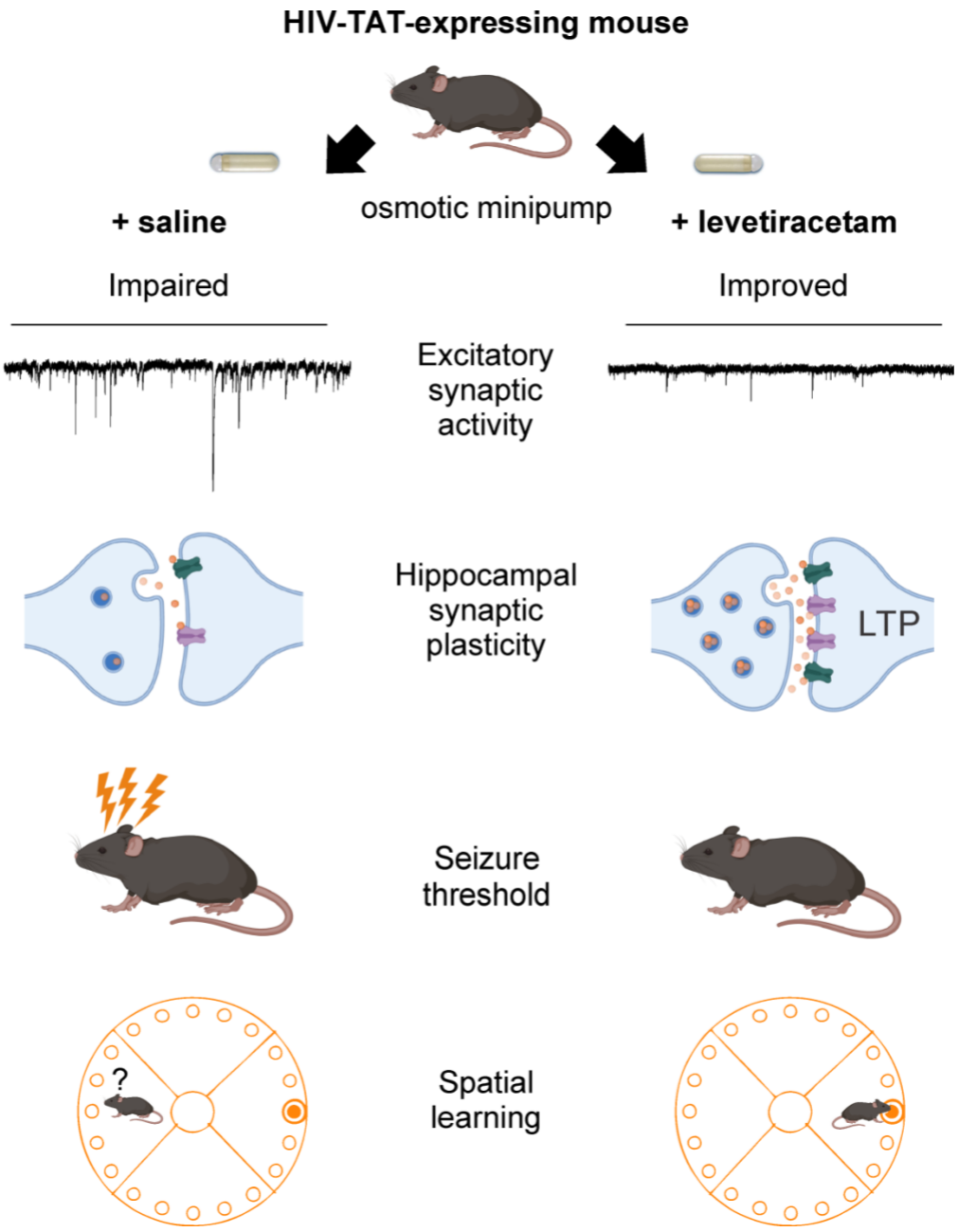
TAT has a wide range of neurotoxic effects, the most damaging of which include magnifying inflammation, impairing the endocannabinoid system, over-activating NMDA receptors, and upregulating inhibitory signaling. In addition, TAT aids the HIV virus in attracting and entering uninfected immune cells, supporting its continued replication. Therefore, a treatment specifically against TAT's neurotoxicity deserves more investigation.

Overall, the modifications to the immune system, endocannabinoid system, NMDA receptors, and inhibitory signaling create an environment conducive to excitatory dysfunction. TAT has also been shown to directly manipulate glutamate signaling and cycling. As a result, TAT has been implicated in causing epileptic activity, although there is limited research. This is supported by the evidence that PLWH have seizures five times more than the general population. Additionally, epileptic activity has been demonstrated to be pathogenic in Alzheimer's disease (AD), which bears many similarities to HAND pathology. Excitingly, not only could this activity be reversed in AD-model mice, but cognition was also preserved using an anti-epileptic drug, levetiracetam (LEV). Since PLWH are at risk for developing seizures that may worsen cognition, LEV could be used as a protective strategy against seizures and cognitive decline.

Here, I show for the first time that LEV prevented synaptic and cognitive impairments that develop in a TAT-expressing mouse model. Inducible TAT (iTAT) transgenic male mice had an increased frequency of spontaneous excitatory post-synaptic currents (sEPSCs) in hippocampal slice recordings. Furthermore, iTAT mice had impaired long-term potentiation (LTP), a form of synaptic plasticity that underlies learning and memory. Two-week administration of LEV through osmotic minipumps prevented both impairments. Additionally, kainic acid induced a higher maximum behavioral seizure score, longer seizure duration, and a shorter latency to first seizure, consistent with a lower seizure threshold in iTAT mice. Acute LEV administration reduced seizures in control and iTAT mice. Lastly, iTAT mice showed cognitive impairments in the Barnes maze that were prevented by chronic LEV administration.

Thus, a TAT-induced increase in glutamatergic synaptic activity drives functional deficits in this model of HAND.

LEV not only prevented aberrant synaptic activity in iTAT mice, but also restored cognitive function. LEV provides a pharmacological approach to prevent neurodegenerative processes and is a promising strategy to afford neuroprotection in HAND. This study supports further investigation of the use of LEV for clinical neuroprotection in HAND patients.



Graphical Abstract. The HIV-TAT transgenic mouse recapitulates cognitive deficits seen in HIV-associated neurocognitive disorder, a disease for which there is no treatment. Administration of levetiracetam, an antiepileptic drug, to mice expressing TAT prevented an increase in excitatory synaptic activity and deficits in synaptic plasticity, protected from severe seizures, and improved spatial learning.

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List of Abbreviations

2-AG: 2-arachidonyl-glycerol
AEA: anandamide
AEDs: anti-epileptic drugs
AIDS: acquired immunodeficiency syndrome
AD: Alzheimer's disease
ANOVA: analysis of variance
BBB: blood-brain barrier
BM: Barnes maze
cART: combined anti-retroviral therapy
CB1R: cannabinoid 1 receptor
CB2R: cannabinoid 2 receptor
CNS: central nervous system
CSF: cerebrospinal fluid
eCB: endocannabinoid
EEG: electroencephalogram
GABA: gamma amino butyric acid
HAND: HIV-associated neurocognitive disorder
HD: Huntington's disease
HIV: Human immunodeficiency virus
LEV: levetiracetam
LTP: long-term potentiation
MMSE: mini-mental state exam
NMDARs: N-methyl-D-aspartate receptors
NOR: novel object recognition
PD: Parkinson's disease
PLWH: people living with HIV
sEPSC: spontaneous excitatory post-synaptic current
sIPSC: inhibitory post-synaptic current
SV2A: synaptic vesicle protein 2A
TAT: transactivator of transcription

Chapter One: Introduction

I: HIV

Human immunodeficiency virus-1 (HIV) is a deadly pathogen that inevitably leads to acquired immunodeficiency syndrome (AIDS) and death from opportunistic infections (Broder and Gallo, 1984). Over 40 million people have died from AIDS-related deaths since the start of the HIV epidemic in 1981 (UNAIDS, 2023). Today, there are still 39 million people living with HIV (PLWH) (WHO, 2023), and despite intervention with antiretroviral drugs, the pandemic is not over.

The exceedingly high number of AIDS-related deaths during the height of the AIDS epidemic quickly prompted research into antiviral drugs that could slow the disease. Zidovudine was first approved for use in 1987 (Kemnic and Gulick, 2023), but long-term prognosis did not improve until the introduction of combined anti-retroviral therapy (cART) in 1996 (Ghosn et al., 2018). PLWH were started on complicated antiretroviral dosing regimens that prolonged their lifespan, but it did not always work for treatment-resistant HIV. Nucleoside reverse transcriptase inhibitors introduced in the early 2000s and the integrase inhibitors used today proved successful even in treatment-resistant patients and reduced disruption of day-to-day life with daily rather than hourly medicine (Tseng et al., 2015). Today, dual therapies (with two drugs rather than four) are even more effective and only require monthly dosing (Taki et al., 2022). cART has since been shown to provide massive preventative benefits in early HIV infection and is now the mainline treatment for HIV (Günthard et al., 2016). The implementation of cART marked a therapeutic milestone that transformed HIV into a manageable condition.

As cART increased the lifespan of PLWH, however, new symptoms emerged. PLWH began experiencing problems with memory, processing, and executive function (Ances and Ellis, 2007). Some individuals even developed dementia. Much was unknown at the time, but a new disease under HIV was recognized – HIV-associated neurocognitive disorder (HAND).

II: HAND

HAND currently affects about half of PLWH; the risk of HAND increases with age (Cherner et al., 2004) and the number of aging PLWH is also increasing (Eggers et al., 2017), highlighting the need to support an aging HIV-positive population. HAND symptoms range from asymptomatic neurocognitive impairment, where PLWH would only know they have it from poor performance on a cognitive test, to HIV-associated dementia (Saylor et al., 2016). HAND and its severity are diagnosed with a battery of seven examinations that measure verbal fluency, executive functioning, processing, learning, recall, working memory, and motor skills (Woods et al., 2004; Antinori et al., 2007). Once PLWH have asymptomatic neurocognitive impairment, they are 2-6 times more likely to eventually develop more severe types, which can happen rapidly (Grant et al., 2014). HIV-associated dementia can be extremely debilitating; like Alzheimer's disease (AD), patients with HIV-associated dementia struggle with communication and day-to-day tasks and often cannot live independently (Xia et al., 2011). HIV-associated dementia is also correlated with shortened lifespans (Navia et al., 1986; Sevigny et al., 2007). Despite this, there is no current treatment available for HAND (Saylor et al., 2016).

It remains unclear how HAND manifests. HAND can develop with or without treatment and its prevalence has not changed despite cART administration (Saylor et al., 2016). As such, it was first considered that the antiretrovirals themselves, originally the gold standard for preventing AIDS, may exacerbate cognitive decline due to their necessary CNS penetrance (Robertson et al., 2012; Underwood et al., 2015). Additional evidence was mounting that even with virologically controlled HIV, viral reservoirs remained in hard-to-reach areas, like the brain, and could not be eliminated (Churchill et al., 2016; Asahchop et al., 2017). The idea that latent viruses can continue to cause damage, even decades after the original insult, was not fully understood at the time, but there is now increasing evidence that HIV is not unique in this respect. The Epstein-Barr virus can lead to multiple sclerosis and tumorigenesis (Bar-Or et al., 2022; Bjornevik et al., 2022; Soldan and Lieberman, 2023) and, in the most intensive study thus far, COVID-19 infection induces a plethora of long-term sequelae, including neurological and cardiovascular insults (Monje and Iwasaki, 2022); the effects that COVID-19 infection has on the body decades later remain to be fully realized. More speculative work suggests that the influenza and herpes simplex viruses may also play a role in the development of Parkinson's disease (PD) and Alzheimer's disease (AD), respectively (Dobson and Itzhaki, 1999; Takahashi and Yamada, 1999). Clearly, viruses can persist beyond the initial infection, and this is likely the cause of HAND development.

III: HAND pathogenesis

Infectious agents that lay dormant in protected reservoirs have a common strategy: entering the brain, which allows escape from the immune system (De Chiara

et al., 2012). HIV is a member of the lentivirus family, one of the first examples to be identified in which a virus can enter privileged sites through a “Trojan horse” mechanism: infected leukocytes can slip through the cracks of a compromised blood-brain barrier (BBB) (Haase, 1986). The BBB is one of the most important protections against inflammatory insult; it relies on impenetrable tight junctions between astrocytes to prevent neurotoxins and pathogens from entering the brain (Begley and Brightman, 2003). BBB disruption is highly associated with neuroinflammation and neurodegenerative disease (Sweeney et al., 2018)

The integrity of the BBB becomes compromised early on in HIV infection (Mackiewicz et al., 2019) through disruption of adhesion molecules (Roberts et al., 2010) and proteins that maintain tight junctions (Persidsky et al., 2006a). This allows infiltration of infected monocytes and macrophages (Strazza et al., 2011; András and Toborek, 2013), leukocytes (Persidsky et al., 2006b), as well as accumulation of amyloid-beta, the protein primarily responsible for AD pathology (András et al., 2010). Once in the brain, infected monocytes can then infect microglia through glycoprotein CD4 and co-receptors CXCR4 and CCR5, where it resides permanently in viral reservoirs (Choe et al., 1996; Feng et al., 1996; Albright et al., 1999). Neurons express CXCR4 (Bonavia et al., 2003), but because they lack CD4 expression, HIV does not directly infect neurons (Gonzalez-Scarano and Martin-Garcia, 2005). Ultimately, BBB disturbances lead to microglial activation and chronic microgliosis, which directly cause neurotoxicity and neurodegeneration (Lu et al., 2011; Mackiewicz et al., 2019). As a result, neuroinflammation is thought to contribute significantly to HAND. Despite this, there is no HAND-specific treatment. The only current approach is continuing

antiretroviral therapy to keep viral levels as low as possible (Churchill et al., 2016). A recent study demonstrated the ability of anti-inflammatory drugs teriflunomide and monomethylfumarate to dampen inflammation in neuronal cultures (Ambrosius et al., 2017), but clinical trials with various anti-inflammatories have been unsuccessful at universally decreasing inflammation (Lv et al., 2021). One of the more promising treatments thus far has been medical cannabis, due to its ability to specifically attack neuroinflammation (Ellis et al., 2020), but its concurrent psychoactive properties make it potentially contraindicated for PLWH, who often are already more susceptible to mental health problems (Lv et al., 2021). As such, decreasing neuroinflammation may alleviate some aspects of HAND, but it is currently not a complete treatment. The complex interactions between HIV and the brain suggest there are additional mechanisms at play that require more scrutiny.

Consequently, research has focused on proteins released in HIV infection that incidentally harm neuronal function. Once the virus is tucked away in microglia and astrocytes, it can continually produce and release HIV-associated proteins; the most virulent of these include Viral protein R (Vpr), Negative regulatory factor (Nef), envelope protein gp120 (gp120), and transactivator of transcription (TAT). These proteins have been implicated as potential contributors to HAND pathology: high Vpr levels in the cerebrospinal fluid are associated with cognitive impairments (Huang et al., 2000) and apoptosis (Patel et al., 2000); Nef aids in BBB disruption (Sporer et al., 2000) and therefore initial HIV infection, as well as apoptosis in neuronal cultures (Trillo-Pazos et al., 2000); and gp120 induces inflammation through IL-1β release (Green and Thayer, 2019; He et al., 2020) and microgliosis (Toggas et al., 1994).

Here, I will focus on TAT, which is the major driver of excitotoxicity, synaptic damage, and neurodegeneration in HAND. This is because TAT is found in the serum of PLWH at a concentration of 2ng/mL – 40ng/mL, even in those with virologically suppressed HIV (Xiao et al., 2000); cART does not stop TAT transcription or synthesis (Ajasin and Eugenin, 2020). TAT is the only HIV-associated protein that conclusively persists in the presence of cART, so it likely drives HAND pathology (Cirino and McLaughlin, 2021). Indeed, animal models of TAT recapitulate both the physiology and symptomology of HAND (Kim et al., 2003; Carey et al., 2012; Fitting et al., 2013; Raybuck et al., 2017). TAT can also reactivate latent HIV (Ajasin and Eugenin, 2020) and induce T-cell apoptosis, a steppingstone to developing AIDS (Campbell et al., 2004). Therefore, therapeutic approaches for both HIV and HAND must focus on combating the effects of TAT.

IV: TAT in HIV and HAND

Despite HIV's high recombination frequency, TAT is a well-conserved protein in the HIV genome; it is essential for accelerating HIV gene transcription (Chen, 1986). TAT is a *trans*-acting transcription factor that interacts with the *cis*-acting sequence, or *trans*-acting response element (TAR), within the long-terminal repeat (LTR) section of HIV RNA (Rosen et al., 1985), fast-tracking HIV gene expression by 50-fold (Kao et al., 1987). TAT ensures this eruption of viral transcription by employing three major strategies – transcriptional initiation, anti-termination prevention, and stabilization of elongated transcripts (Kao et al., 1987; Rice and Mathews, 1988; Laspia et al., 1989).

Incidentally, TAT also has a high number of interactions with the host. This is likely due to TAT's ability to recruit other transcriptional regulators and enrich TAR-like binding sites within the human genome (Marban et al., 2011; Reeder et al., 2015). To do this, TAT must first devise a way for cells to take it up: TAT binds to lipoprotein receptor-related protein, proteoglycans, and the CXCR4 receptor, among others, to facilitate its internalization into a wide variety of cells (Liu et al., 2000; Tyagi et al., 2001; Fittipaldi et al., 2003). TAT so expertly invades cells that its core peptide region is used in research unrelated to HIV to deliver molecular cargo (Zou et al., 2017). As a result, TAT changes the expression of approximately 500 host genes through direct binding and indirectly alters the expression of 1,500 others (Reeder et al., 2015). Of note, TAT increases the expression of CXCR4 and CCR5, the main receptors through which HIV infects and interacts with the immune system, making cells more susceptible to HIV infection (Huang et al., 1998). Together, these ubiquitous interactions achieve TAT's primary goals of accelerating HIV gene expression and infiltration.

Beyond transcriptional modifications, TAT has a myriad of detrimental interactions with the CNS that majorly contribute to HAND pathogenesis. Here I will highlight the TAT-modulated pathways that contribute most heavily to HAND neurotoxicity: manipulation of the host immune system to magnify inflammation, decreasing the threshold for excitability via NMDA receptors, and impairment of the endocannabinoid (eCB) system.

IV(a): TAT-induced inflammation and indirect effects

TAT creates a neuroinflammatory environment through many indirect insults to the CNS. In early infection, TAT activates peripheral myeloid cells that phagocytose synapses (Lu et al., 2011). This not only disrupts normal architecture in the short-term, but also causes enduring inflammation and inflammatory consequences, like long-term microgliosis (Lu et al., 2011) and astrocytic dysfunction (Cirino and McLaughlin, 2021). Specifically, TAT infects and manipulates astrocytes to increase inflammation (Conant et al., 1998; El-Hage et al., 2005). Inflammatory promoters and genes, such as MCP-1, E-selectin, and IL-6 are upregulated (András et al., 2008). Both microglial activation and TAT itself induce release of pro-inflammatory cytokines TNF-α, IL-10 (Ben Haij et al., 2013) and IL-1β (Chivero et al., 2017) and chemokines that attract uninfected T-cells (Kutsch et al., 2000). This begins and maintains the cascade of damage to normal excitatory and inhibitory signaling.

Other miscellaneous effects of TAT that aid in neurotoxicity include downregulation of BDNF, a protein crucial for health and neurogenesis that is also pathologically low in AD and PD (Liu et al., 2018); association with fewer neural progenitor cells (Fan et al., 2016), which limits genesis of new neurons; morphology changes that cause CD11+ immune cells to become hypertrophic and wrap around neurons (Lu et al., 2011); shifting redox state to pro-oxidative (Westendorp et al., 1995); and causing global apoptosis (New et al., 1997; Eugenin et al., 2007). These widespread neurological insults, coupled with heighted neuroinflammation, set the stage for excitatory damage.

IV(b): TAT damage to excitatory and inhibitory pathways

Glutamate is a major excitatory neurotransmitter that TAT directly disrupts. Normally, glutamate released from excitatory terminals binds to N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) on the postsynaptic membrane (Blanke and VanDongen, 2009). Extra glutamate in the synapse is managed by neuronal and astrocytic reuptake through glutamatergic cycling (Rowley et al., 2012; Zhou and Danbolt, 2014). TAT interferes with this process and increases glutamatergic load. TAT not only increases spontaneous glutamate release (Brailoiu et al., 2008; Jacobs et al., 2019), but also decreases glutamate reuptake, allowing for glutamate levels to remain heightened in the synapse (Saylor et al., 2016; Cirino and McLaughlin, 2021). Indeed, astrocytic infection with TAT is sufficient to recapitulate HAND physiology and symptomology (Zhou et al., 2004; Fan et al., 2011). Glutamate levels are also five times higher in the cerebrospinal fluid (CSF) of PLWH compared to their HIV-negative counterparts (Ferrarese et al., 2001). More specifically, glutamate levels are higher in PLWH with HAND compared to HIV patients with no cognitive impairments (Cassol et al., 2014), suggesting a critical role of glutamate and excitotoxicity in HAND development.

Ultimately, through glutamate, TAT increases the probability of NMDAR and AMPAR overactivation. Proper glutamatergic signaling must be kept in careful balance, as NMDAR dysfunction has been implicated in many disease states: NMDARs have reduced expression in schizophrenia (Pilowsky et al., 2006) and are overstimulated in major depressive disorder (Adell, 2020) and AD (Parsons and Raymond, 2014). In HAND, TAT activates and potentiates NMDARs (Song et al., 2003; Krogh et al., 2014; Green and Thayer, 2016). Further, TAT alters the expression of protein phosphatase 1,

which normally functions to suppress NMDARs (Liu et al., 2018). The resulting NMDAR overactivation initially causes a toxic influx of calcium (Kim et al., 2008; Xu et al., 2016) and later leads to protective downscaling of excitatory synapses (Kim et al., 2008; Shin et al., 2012; Bertrand et al., 2013). The overactivation of excitatory synapses induces a homeostatic response that upregulates inhibitory synapses (Hargus and Thayer, 2013). Not only are synapses physically changed, but they are also functionally different, although the evidence for what exactly TAT does to inhibitory signaling is mixed – TAT has been found to both increase spontaneous inhibitory signaling (Brailoiu et al., 2008) and decrease potassium-induced GABA release (Musante et al., 2010).

One of many fallback mechanisms that exist to protect the brain from excessive excitatory signaling is the eCB system, which is also damaged by TAT. The eCB system consists of endogenously produced cannabinoids (endocannabinoids) 2-arachidonyl glycerol (2-AG) and anandamide (AEA) that are synthesized on-demand post-synaptically and bind to pre-synaptic cannabinoid receptors 1 (CB1R) and 2 (CB2R) to dampen excitation and protect from seizures (Sugaya and Kano, 2018; Wu et al., 2019; Cristino et al., 2020). TAT not only impairs CB1R function, but also the efficacy of 2-AG to exert its inhibitory effects (Wu and Thayer, 2020). Additionally, depolarization-induced suppression of excitation (DSE), a type of short-term inhibition employed by the eCB system, is damaged by TAT, while depolarization-induced suppression of inhibition (DSI) remains intact (Wu and Thayer, 2020). This supports an excitatory/inhibitory imbalance.

Overall, the modifications that TAT makes to the immune system, eCB system, and inhibitory signaling support an environment conducive to excitatory dysfunction. By

increasing neuroinflammation and hijacking NMDAR signaling, TAT decreases the threshold for excitability. This is supported by limited but compelling evidence of a hyperexcitable phenotype in HAND: more action potentials are fired in response to the same stimulus in the presence of TAT (Brailoiu et al., 2008) and TAT-expressing mice have increased susceptibility to seizures induced by kainate, an analog of glutamate (Zucchini et al., 2013). Moreover, seizures occur significantly more often in PLWH than the general population (Kellinghaus et al., 2008; Ssentongo, 2019). This demands further investigation into how epileptic activity may contribute to HAND.

V: Pathogenicity of epileptic activity

Despite increased seizure prevalence in HIV, research on epileptic activity in HAND is lacking. Some limited early studies demonstrated that the severity of HIV dementia correlated with the severity of EEG abnormalities (Parisi et al., 1989). These included slowing of delta and theta waves (Gabuzda et al., 1988), which are associated with mild dementia when intermittent and severe dementia when continuous (Baldeweg and Gruzelier, 1997). PLWH that had not received antiretroviral treatment also had increased quantitative electroencephalogram (qEEG) alpha and theta wave amplitudes, which indicates an imbalance of excitatory and inhibitory signaling (Baldeweg and Gruzelier, 1997).

More recent research has shown that the longer patients have HIV, the more likely they are to have associated EEG and neuroimaging abnormalities (Siddiqi et al., 2015). However, this research was conducted in HIV patients with new-onset seizures (due to lack of intervention with antiretrovirals) and a quarter of the patients died during

the study, so this cohort is not likely to be an accurate representation of all PLWH. Although these initial studies provide valuable insight into the damage HIV can do when left untreated, there is still a lack of understanding in how to prevent seizures from happening in the first place. Most studies can only tell us what we already know about advanced disease stages, and yet there must be something detectable at the molecular level that precedes seizures in PLWH. An EEG biomarker for HAND is desperately needed but still does not exist (Antinori et al., 2007; Ishii and Canuet, 2014).

Evidence of epileptic activity being pathogenic in AD may pave the way for preventative measures and treatments for HAND. Multiple AD-model mice have subclinical epileptiform activity, which is characterized as fast spike deflections on EEGs without overt seizures (Palop et al., 2007; Vogt et al., 2011; Sanchez et al., 2012). Additionally, not only were *ex vivo* brain slices from AD mice shown to have impaired synaptic plasticity, but AD mice were also significantly impaired in learning and memory tasks requiring the hippocampus (Sanchez et al., 2012). Following this, the same subclinical activity was found in 42% of AD patients (Vossel et al., 2016). AD patients with epileptiform activity performed significantly worse on the mini-mental state exam (MMSE), which tests basic cognitive abilities such as concentration, short-term memory, and visual-spatial memory, than patients that did not (Vossel et al., 2016). Seizures in general are also more common in AD than the overall population (Mendez and Lim, 2003). Seizures in AD are associated with worse prognosis (Irizarry et al., 2012; Vossel et al., 2016; Sen et al., 2018); they occur before overt neurodegeneration, and therefore, likely contribute to it (Palop and Mucke, 2016).

The idea that epileptic activity contributes to pathogenesis is still emerging but gaining traction in similar neurodegenerative disease states. Epilepsy is one such disease, in which chronic seizures cause widespread damage that mimics hallmarks of neurodegeneration, including BBB disruption, apoptosis, and neuroinflammation (Farrell et al., 2017). Interictal epileptiform activity, where single spikes occur between seizures, has definite impacts on short-term cognition, but whether this activity must be frequent to cause long-term cognitive disturbances in people with epilepsy is still debated (Sánchez Fernández et al., 2015). What is clear is that having epilepsy for longer periods of time is associated with greater white matter deterioration (Slinger et al., 2016) and controlling seizures leads to better cognitive outcomes (Braun, 2017).

Similarly, a recent study in PD found that patients with visual hallucinations had epileptiform activity on EEGs, while patients without visual hallucinations did not (Fry et al., 2021). The onset of visual hallucinations is the threshold for psychosis in PD, suggesting that epileptiform activity is associated with worsening disease prognosis in PD. With this limited information, it has been challenging to determine whether epileptic activity is simply another symptom of neurodegeneration or whether it plays a role in pathogenesis. Research in Huntington's disease (HD) may provide insight: interictal spikes were present on EEG recordings in all HD-model mice, with a few having full seizures (Pignatelli et al., 2012). The mice used in this study were 16-26 weeks old, equivalent to approximately 20-35 years old for humans (Flurkey K, 2007). The average age of onset for HD is 45 years old (Ross et al., 2014). Cognitive impairments in HD are estimated to begin 9-15 years before diagnosis; these impairments are usually mild and often found incidentally on cognitive tests (Stout et al., 2011), much like in HAND. With

this information, it is plausible that subclinical epileptic activity occurs in HD in the years leading up to diagnosis and contributes to worsening disease progression. However, more research on epileptic activity and biomarkers in the preliminary stages of neurodegenerative disease before irreversible pathology has occurred are desperately needed.

How exactly epileptic activity contributes to worsening cognition is poorly understood. Current theories include physical degeneration of excitatory terminals and compensatory upregulation in inhibitory signaling. In epilepsy, it is practically impossible to determine whether cognitive deficits are caused by the seizures themselves, the resulting physiology changes, interictal spikes, the regimen of high-dose anti-epileptic drugs (AEDs) necessary to control seizures, or some combination therein (Braun, 2017). In AD-model mice, kainate injection increased expression of neuropeptide Y and GABAergic sprouting; these neurophysical abnormalities were also found in untreated AD-model mice (Palop et al., 2007), suggesting that even a single seizure mimics AD-like neurophysiology. Upregulated inhibition has been shown to impair synaptic plasticity (Kleschevnikov et al., 2004). However, more research must be done to conclude how intermittent epileptic activity leads to downstream neurotoxicity and degeneration in HAND.

The TAT-induced alterations to the immune system, the eCB system, and NMDARs allows the balance between excitation and inhibition to be upended and creates the opening necessary for epileptic activity. Additionally, the increased glutamatergic levels in the CSF of HAND patients supports an issue with glutamatergic cycling, which is well-established to be hijacked by TAT. It also points to epileptic

activity accelerating cognitive impairment, as glutamate levels are elevated in the CSF of epilepsy patients following seizures (Sarlo and Holton, 2021). Additionally, the cytokines that TAT increases (TNF-α, IL-1β) are found at high levels both before and after seizures (Soltani Khaboushan et al., 2022). How epileptic activity may contribute to HAND is yet unknown, but epilepsy in HIV is correlated with worse cognitive outcomes (Siddiqi et al., 2015). Therefore, epileptic activity must be addressed to combat, or at the very least, slow cognitive decline in HAND.

VI: Cognitive protection provided by AEDs

If epileptic activity is occurring and accelerating progression of neurodegenerative disease, AEDs might protect from excitotoxic damage. Valproate protects from amyloid-beta toxicity *in vitro* (Mark et al., 1995), but surprisingly few studies have used AEDs to treat AD, and these showed little success (Herrmann et al., 2007). Interest in AEDs for neuroprotection declined until a study using an AD-model mouse found levetiracetam (LEV) attenuated subclinical epileptiform spiking and reversed cognitive deficits (Sanchez et al., 2012). Intriguingly, it was the only AED that was able to do so; ethosuximide, gabapentin, phenytoin, pregabalin, valproate and vigabatrin all failed to attenuate the electrographic spiking.

LEV's unique success in cognitive protection is likely due to its alternative mechanism. Most first-generation AEDs inhibit voltage-gated sodium channels, potassium channels, or calcium channels (Sills and Rogawski, 2020), while LEV works intracellularly. LEV's primary target is synaptic vesicle protein 2A (SV2A) (Lynch et al., 2004), a protein that regulates readily releasable pools and influences vesicle priming

(Wu et al., 2023). Ultimately, the interaction of LEV and SV2A attenuates glutamate release (Mruk et al., 2015) while sparing normal glutamatergic synaptic transmission (Bradberry and Chapman, 2022), but how this occurs remains elusive; it is not known whether LEV inhibits or increases SV2A's actions.

LEV is already FDA-approved and prescribed to PLWH with severe epilepsy (Siddiqi and Birbeck, 2013), but it is unknown if it provides neuroprotection in HAND. Here, I used LEV as a tool to determine the role of hyperexcitability in HIV-associated cognitive decline.

VII: Summary and rationale for thesis

Despite extensive research, the advent of cART has not changed HAND prevalence and there is no treatment specifically to combat HAND (Robinson-Papp and Saylor, 2021). Once PLWH have asymptomatic neurocognitive impairment, they are much more likely to develop more severe types over time. As such, it is imperative to identify biomarkers and determine causes of HAND to prevent cognitive decline. However, it is still unclear how HAND manifests. The HIV protein TAT is thought to be involved due to its wide-ranging effects on the human immune system and excitatory and inhibitory balance.

There is compelling evidence that TAT induces subtle changes in glutamatergic cycling, which contribute to subclinical epileptic activity. It is unknown whether this activity exists in HAND and if it is pathogenic in HAND development. The emerging hypothesis that epileptic activity contributes to neurodegeneration is supported by evidence from its pathogenicity in HD and AD disease development. I hypothesize that

TAT causes epileptic activity that drives cognitive decline in HAND. This hypothesis predicts that the use of LEV would prevent epileptic activity and resulting cognitive decline.

Here, I used a transgenic mouse model of HAND with doxycycline-inducible expression of TAT (iTAT). I found that iTAT mice had heightened spontaneous excitatory signaling, impaired synaptic plasticity, and cognitive deficits, in agreement with previous use of this model (Carey et al., 2012; Fitting et al., 2013). Here, we showed for the first time that administration of an anti-epileptic drug, LEV, prevented these deficits. As a result, the role of epileptic activity in neurodegeneration and how it may be prevented by LEV deserve more study to potentially slow or halt cognitive decline in PLWH.

Chapter Two

Levetiracetam prevents neurophysiological changes and preserves cognitive function in the HIV-1 TAT transgenic mouse model of HIV-associated neurocognitive disorder

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I: Introduction

Worldwide, there are 39 million people living with HIV-1 (PLWH) (https://www.who.int/data/gho/data/themes/hiv-aids#), half of whom meet the criteria for HIV-associated neurocognitive disorder (HAND) (Heaton et al., 2010). HAND symptoms range from subclinical impairments to dementia (Alford et al., 2022; Nightingale et al., 2023). Although combined anti-retroviral therapy has greatly improved the quality of life for PLWH, the prevalence of HAND has not changed (Robinson-Papp and Saylor, 2021). Currently there is no treatment for HAND. In this study we examine the contribution of excess excitatory synaptic activity to cognitive impairment in HAND and evaluate an approach to prevent it. We used a mouse model in which expression of the HIV-1 protein TAT (trans-activator of transcription) in the CNS produces synaptic and cognitive impairments (Kim et al., 2003; Fitting et al., 2013) similar to those observed in patients with HAND (Saylor et al., 2016).

The HIV protein TAT is shed by infected monocytes and microglia (Nath et al., 1999; King et al., 2006) and is a potent neurotoxin (New et al., 1997). After HIV DNA integrates into the host genome, TAT expression and secretion continue in the CNS of virologically-controlled PLWH as indicated by elevated levels in the cerebrospinal fluid and the uptake of TAT by uninfected bystander cells (Johnson et al., 2013; Donoso et al., 2022). TAT is thought to contribute to HAND by an excitotoxic mechanism (Green et al., 2018) that elevates intracellular Ca^{2+} concentration (Nath et al., 1996; Haughey et al., 2001; Hu, 2016) and increases glutamatergic signaling (Eugenin et al., 2007; Green and Thayer, 2016; Jacobs et al., 2019), leading to synapse loss and cognitive impairment (Fitting et al., 2013; Raybuck et al., 2017). TAT-induced synaptic changes

precede overt cell death (Kim et al., 2008; Green et al., 2019) and synapse loss correlates with cognitive decline in HAND (Ellis et al., 2007). Synaptic changes, including the loss of excitatory synapses, are reversible (Shin et al., 2012).

Increased excitatory synaptic drive contributes to network dysfunction in neurodegenerative disorders (Verma et al., 2022). Hyperexcitability has been noted both in animal models and patients with HAND. Specifically, systemic expression of HIV-1 TAT increased the severity of evoked seizures in mice (Zucchini et al., 2013) and PLWH have an increased risk of seizures (Kellinghaus et al., 2008). Such hyperexcitability may directly contribute to both neurodegeneration and cognitive deficits in HAND patients. Epilepsy patients can show neurodegeneration and cognitive decline (Casillas-Espinosa et al., 2020). Epileptiform activity can also occur before overt neurodegeneration in Alzheimer's disease (AD), and therefore, likely contributes to cognitive decline (Palop and Mucke, 2016). As such, it is possible that hyperexcitability occurs in PLWH and contributes to cognitive decline in HAND.

If hyperexcitability accelerates progression of neurodegenerative disease, antiepileptic drugs (AEDs) might protect from excitotoxic damage. Valproate protects from amyloid-beta toxicity *in vitro* (Mark et al., 1995), but surprisingly few studies tested AEDs in AD, and these showed little success (Herrmann et al., 2007). Interest in AEDs for neuroprotection declined until a study using an AD-model mouse found levetiracetam (LEV) attenuated subclinical epileptiform spiking and reversed cognitive decline (Sanchez et al., 2012). Intriguingly, it was the only tested AED that was able to do so. This is possibly due to LEV's unique mechanism. LEV binds to the synaptic vesicle protein SV2A to inhibit excess glutamate release while sparing normal

glutamatergic synaptic transmission (Bradberry and Chapman, 2022; Contreras-García et al., 2022). LEV is well-tolerated by PLWH (Siddiqi and Birbeck, 2013), but whether it provides neuroprotection in HAND is unknown.

In this study, we used LEV as a tool to determine the role of excess glutamatergic synaptic activity in a mouse model of HAND and determined its potential to restore synaptic and cognitive function. TAT expression increased glutamatergic signaling, impaired synaptic plasticity, lowered seizure threshold, and impaired learning and memory, all of which were prevented or reduced by LEV, suggesting a pathological increase in glutamatergic activity drives cognitive impairment in HAND.

II: Materials and Methods

Animals

All mice (8-14 weeks old) were housed in the University of Minnesota animal care facility and treated in accordance with the US National Institutes of Health guidelines under a protocol approved by the Institutional Animal Care and Use Committee. Male mice were used in this study as TAT effects are less robust in female mice (Hahn et al., 2015). Mice were group-housed with a 12h:12h light/dark cycle and food and water provided ad libitum. Development of the doxycycline (DOX) inducible, brain targeted transgenic mouse expressing HIV-1 TAT_{1-86} (iTAT) was described previously (Kim et al., 2003). These GFAP-rtTA-TRE/Tat mice backcrossed onto a C57BL/6J background were kindly gifted from the lab of Dr. Jay McLaughlin and were bred in-house (Carey et al., 2012). Wild-type (WT) mice from Jackson Labs (C57BL/6J) were used as controls. Experimental iTAT and WT male mice were administered DOX (100 mg/kg) by IP injection (0.3 mL/30 g) once a day for 7 d. For EEG and kainic acid (KA) experiments, due to varying degrees of seizure susceptibility reported for different strains of C57BL/6 mice (Bankstahl et al., 2012), iTAT mice injected daily with saline (SAL) for 7 d were used as controls. TAT expression was confirmed using qRT-PCR as previously described (Zhang and Thayer, 2018) with TAT-specific primers 5'-ATG GAG CCA GTA GAT CCT AG-3' and 5'-GGG TTG CTT TGA TAG AGA AAC TTG-3' (Mediouni et al., 2015b); TAT was undetectable in WT and uninduced iTAT mice. Mouse GAPDH was used as a loading control and analyzed using the following primers: 5'-CTC AGT GTA GCC CAG GAT GC-3' and 5'-ACC ACC ATG GAG AAG GCT GG-3'.

Materials

Materials were obtained from the following sources: halothane was obtained from Santa Cruz Biotechnologies (sc-251705; Dallas, TX); isoflurane from Dechra Veterinary Products (Overland Park, KS); saline from Nova-Tech, Inc. (Grand Island, NE); levetiracetam (LEV) from Thermo-Fisher Scientific (462120050; Waltham, MA); picrotoxin from Tocris Biosciences (1128; Bristol, UK); trizol from Invitrogen (15596018; Waltham, MA). Cesium gluconate (HB4822), DNQX (HB0262), QX-314 (HB1030), and KA (HB0355) were obtained from HelloBio (Princeton, NJ.); Vectashield mounting medium was obtained from Vector Laboratories, Inc. (H-1200; Newark, CA). All other drugs and reagents were obtained through Sigma-Aldrich (St. Louis, MO).

Osmotic minipumps

Mice were implanted with osmotic minipumps 14 d before electrophysiology and behavioral experiments. Briefly, osmotic minipumps (Alzet; Cupertino, CA; model 2004) were filled with sterile LEV (150mg/kg/day) dissolved in 0.9% sterile SAL or SAL alone according to manufacturer's instructions. Mice were injected with meloxicam (5mg/kg) 1 h before surgery and were anesthetized with 1% isoflurane during surgery. Lidocaine was applied to the surgical area before a 0.5-inch incision was made in the scapular region. The subcutaneous space was expanded using a hemostat. Pumps were placed with the opening facing away from the surgical incision site and the incision site stitched with dissolvable sutures. Mice were allowed to recover from surgery for 1 week before beginning DOX injections. Meloxicam injections were given once daily for 3 d following surgery. Plasma LEV concentration was determined by ELISA at the Auburn University

Veterinary Clinical Pharmacology Laboratory from trunk blood collected at the time of brain slice preparation.

Electrophysiology

Mouse brains were collected for electrophysiology experiments 1-2 h after the last DOX injection. Mice were anesthetized with halothane and decapitated. The brain was quickly extracted and placed into ice-cold slicing solution containing (in mM): 100 sucrose, 30 NaCl, 3 KCl, 0.5 $CaCl_2$, 28 $NaHCO_3$, 7 $MgCl_2$, 1.4 NaH_2PO4, and 11 D-Glucose (Holth et al., 2013). Slices were constantly carbogenated (95% O_2 / 5% CO_2). The brain was mounted onto a Leica VT1000 stage submerged in ice-cold slicing solution. Coronal slices were obtained at 400 µm thickness. Slices containing the hippocampus were isolated and placed into a pre-warmed (32^{O} C) and pre-carbogenated chamber filled with artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, 1 $MgCl_2$, 1.25 NaH_2PO4, and 11 D-Glucose for 30 min. The chamber and slices were allowed to equilibrate to room temperature for at least 10 min before beginning experiments. All slice recordings were performed at room temperature in ACSF.

For long term potentiation (LTP) experiments, pipets were pulled with a horizontal micropipette puller (P-87 or P-1000, Sutter Instruments) and filled with ACSF (2-4 MΩ). A Grass S44 stimulator with a SIU-5 stimulus isolation unit (Grass Instruments, West Warwick, RI) was used to deliver voltage pulses at 0.033Hz. Slices were stimulated with a concentric bipolar platinum electrode positioned at the Schaffer collaterals. Recording pipets were placed in the stratum radiatum of CA1, downstream

of the stimulating electrode. Recordings were collected at a sampling rate of 20 kHz and filtered with a 10 kHz low pass Bessel filter. Responses were measured using the slope of the field excitatory post-synaptic potential (fEPSP). The stimulation strength for the baseline was determined by increasing the voltage of the stimulating electrode until a population spike was seen. The stimulation strength was then turned down to half the voltage required to elicit this response and baseline fEPSP responses collected (Abrahamsson et al., 2017). Slices were stimulated at 0.033 Hz for at least 30 min, or until a stable baseline was achieved. If a stable baseline was not achieved after 60 min or the fEPSP slope deviated more than 25% during baseline recording, slices were excluded from experiments. Once a stable baseline was established, a single, 1-s high-frequency stimulation (HFS) was given at 100 Hz to induce LTP. Responses were recorded for at least 50 min after HFS.

For whole-cell patch-clamp recordings, pipets (3.5-6 MΩ) were filled with internal solution. For sEPSC experiments, the intracellular solution contained (in mM): 60 Cs-gluconate, 80 CsCl, 3 NaCl, 1 $MgCl_2$, 10 HEPES, 2 Mg-ATP, and 5 QX-314 (Nemeth et al., 2007). For sIPSC experiments, the internal solution contained (in mM): 140 CsCl, 4 NaCl, 1 $MgCl_2$, 10 HEPES, 3 Mg-ATP, 0.5 EGTA, and 5 QX-314 (Hajos and Freund, 2002). Both internal solutions were adjusted to a pH of 7.25 with CsOH. The osmolarities of internal solutions (285-300 mOsm/L) were adjusted up with sucrose to 10 mOsm/L less than the osmolarity of the external solution (300-315 mOsm/L).

Once the whole-cell conformation was established, cells were held at -65 mV and observed for spontaneous activity. For sEPSC experiments, 50 μM picrotoxin was added to the bath by perfusion. For sIPSC experiments, 50 μM APV and 10 μM DNQX

were added to the bath. The internal solution and external drugs were allowed to reach equilibrium for 5 min before spontaneous events were analyzed. Whole-cell currents were amplified with an Axopatch 200B (Molecular Devices) and digitized with a 1550B Axon Digidata.

Depth EEG Recordings

Mice were group-housed until surgery, then two bipolar twisted microwire electrodes (50 µm diameter stainless steel with polyimide insulation, P1 technologies) for depth electrographic recordings were implanted in the hippocampus. The implant procedure was performed under isoflurane anesthesia with subcutaneous carprofen (5 mg/kg) and local subcutaneous bupivacaine. Electrodes were implanted into the hippocampus and secured to the skull with Metabond acrylic. Seven mice were implanted with one dorsal (from Bregma: A/P -0.2 mm, M/L 0.125 mm, and D/V -0.14 mm) and one ventral (A/P -0.35 mm, M/L 0.25 mm, D/V -0.225 mm) electrode; four mice were implanted with a slightly more posterior and lateral dorsal electrode (A/P -0.26 mm, M/L 0.175 mm, D/V -0.14 mm). Mice were treated with Neo-Predef and perioperative analgesics (ibuprofen, 50-80 mg/kg/day dissolved in drinking water for one preoperative day and three postoperative days). Mice were monitored and allowed to recover for at least 1 week after surgery prior to chronic recording. Implant locations were confirmed with histochemical analysis in a subset of mice (n=3).

Mice were singly housed in clear, square cages with visual and olfactory access to other mice. Patch cables were connected to a Brownlee model 440 amplifier and signals were digitized with a sampling frequency of 1000 Hz (National Instruments). The

hippocampal local field potential (LFP) was acquired online and baseline recordings were obtained for at least 7 days before beginning DOX or SAL injections. Mice were continuously monitored with video cameras.

Depth EEG (LFP) recordings were analyzed using custom MATLAB (Mathworks, Natick, MA) based software (a version of which is available through GitHub: https://github.com/KM-Lab/Electrographic-Seizure-Analyzer; RRID: SCR_016344). Power spectrum density plots were generated using Analyzer (BrainVision, Garner, NC) software. Subclinical epileptiform spike detection was first based on guidelines from (Sanchez et al., 2012), then optimized using multiple individualized factors for each mouse, such as spike width, spike amplitude, distance from other spikes, and noise elimination, using custom software (Zeidler et al., 2018; Stieve et al., 2023).

KA-induced seizures

EEG-implanted mice were injected subcutaneously with 30 mg/kg KA and observed and video-recorded for 2 h post-injection. For behavioral testing, mice without EEG implants were injected subcutaneously with 40 mg/kg KA and observed and video-recorded for 2 h. Mice that received LEV were injected with 200 mg/kg IP 1 h before KA (at the same time as DOX or SAL injection to minimize the number of injections). Following KA injection, mice were assigned a seizure score each minute using an extended Racine scale (Pinel and Rovner, 1978; Krook-Magnuson et al., 2015) (1 = change in behavioral state – arrest or sudden motion; 2 = head nodding; 3 = forelimb clonus; 4 = rearing or clonus on belly or strong hind limb clonus / bucking; 5 = falling or clonus on side; 6 = multiple sequences of rearing and falling or brief jumps; 7 = violent

jumping; 8 = class 7 followed by tonus longer than 5s). From these scores, the latency to first stage 3 seizure and cumulative seizure score were determined. Maximum behavioral scores were verified by a blinded observer.

Histochemistry

At the end of EEG recordings, mice were anesthetized with isoflurane, euthanized by decapitation, and brains were collected for analysis. The dental dam was cut from the skull and was separated as gently and quickly as possible. Once extracted, brains were placed in 4% paraformaldehyde at 4^{O}C for 72 h, then placed in 0.1 mM phosphate buffer (PB) at 4^{O}C until slicing. Brains were sliced at 50 μm in 0.1 mM PB using a Leica VT1000. Slices were placed into a 24-well plate in groups of 4-5 per well. Every third to fourth slice was mounted onto slides and stained with Vectashield mounting medium with DAPI for visualization of electrode tracks.

Novel Object Recognition (NOR)

The novel object recognition (NOR) test was conducted in 5 phases (handling, habituation, familiarization, short-term object memory and long-term object memory tests) over a period of 4 days, starting 1d-3d after the last DOX injection. Mice were habituated to the testing room each day for 30 min before beginning testing. Approximately 24 h after acclimating mice to the experimenter by handling, mice were placed in the center of a square (40 x 40 cm) chamber (ANY-maze; Wood Dale, IL) illuminated by an overhead LED lamp (~30 lux) and allowed to explore for a 10 min habituation period, during which mice were tracked using ANY-maze software (Wood

Dale, IL). Twenty-four hours after habituation, mice were returned to chambers with the inclusion of two identical 50 mL Falcon tubes filled with pink liquid placed 13.33 cm from the edges of the chamber; objects were placed in the top-left and bottom-right quadrant of the chamber. Mice were allowed to explore the chamber and objects for 10 min with activity tracked (familiarization). Object memory was first tested 3 hours after familiarization during which a novel object (3.81 x 3.81 x 3.81 cm black wooden cube) replaced one of the Falcon tubes; mice were allowed 10 min in the chamber with the familiar and novel object. Lastly, 24 hours after the first object memory test, a second test was conducted wherein subjects were placed in chambers for 10 minutes with the Falcon tube replaced by a second novel object (flat culture flask with yellow liquid). For object memory tests, the recognition index, a ratio of [time spent with novel object / (time spent with familiar object + time spent with novel object)], was calculated. Meters traveled and raw object interaction time were also recorded.

Barnes maze

Barnes maze testing was conducted using a 6-day protocol, beginning 48 h after completion of the last NOR test. Mice were habituated to the testing room each day for 30 min before beginning testing. Posters with unique geometric shapes were placed on each wall of the room to provide distinct visual cues during testing. Testing was conducted in the dark under red lights; LED lights were turned on when a mouse began a trial and were turned off as soon as the trial ended. On day 1, mice were habituated to the maze apparatus which consisted of a flat, white, circular platform approximately 1 m off the ground with 20 equally spaced holes located at the edges of the circle (San

Diego Instruments); the closed-cup configuration, in which all non-escape holes are covered from underneath the table, was used. The mouse was placed onto the apparatus by the experimenter and a black box placed over the mouse for 15 s. Once the box was removed, LED lights directly over the maze were brought up to their maximum brightness (~300 lux) and activity tracked using ANY-maze software. Mice were allowed to explore the maze with all holes covered for 60 s, after which they were placed into the escape box, covered with a paper towel, and the LED lights were turned off. After 60 s, mice were returned to their home cage. Mice were then trained to locate the escape hole with 4 trials per day for 4 days. In each trial, mice were placed onto the maze in the same manner as habituation and allowed to explore the maze for 180 s. The latency to escape (when all four paws entered the escape hole) was recorded for each trial; latencies were averaged over the 4 trials each day. If the mouse did not escape within 180 s, they were gently guided by the experimenter until they entered the escape hole. Groups of 3-5 mice were run at a time with at least 15 min between each trial. On day 5, a probe test was conducted, in which mice were placed onto the apparatus in the same manner as acquisition, but the escape box was removed and the hole was covered in the same fashion as the other holes. Mice were allowed to explore the maze for 90 s and time spent in the goal quadrant was recorded. On day 6, a reversal test was conducted, in which the escape box was located 180 degrees opposite to its previous location. Mice were trained for 4 trials and latency to escape was recorded. Search strategy and cognitive score were assessed using Barnes maze unbiased strategy (BUNS) analysis (Illouz et al., 2016). Four trials (4/816) were not included in the BUNS analysis due to tracking errors. The maze and escape box were

cleaned with ethanol and allowed to dry after each trial. The maze was rotated 90 degrees each day to ensure that mice were using spatial memory rather than scent tracking to locate the escape hole.

Experimental design and statistical analysis

Sample sizes for LTP and EEG recordings were based on published work using a similar approach (Sanchez et al., 2012), as were sIPSCs (Baez et al., 2020), kainate-induced seizure experiments (Yang et al., 2023), and the Barnes maze (Carey et al., 2012). For sEPSCs and behavioral tests, power analysis was used to estimate the group size needed to detect a true difference 80% of the time at 0.05 significance. Pilot data from NOR and Barnes maze studies were used to estimate variance and effect size.

Spontaneous EPSCs were analyzed using MiniAnalysis software (Kim et al., 2021). Epochs of 2.5-5 min after the 5 min drug application were used for analysis. If the access resistance (R_A) and spontaneous frequency were stable for at least 2.5 min, the recording was included in analysis. Cells were only included for analysis if the initial R_A was < 35 MΩ and did not change by more than 25 % over the course of the recording. LTP experiments were analyzed using pCLAMP v.11.0.

Statistical analyses were performed using Prism 8 (GraphPad Software). All experiments were tested for normal Gaussian distribution with the D'Agostino-Pearson test and homogeneity of variance was determined with Spearman's test for heteroscedasticity. For experiments with two groups, a Student's t-test was performed. For experiments with 3 or more groups, a one or two-way ANOVA was performed with

Tukey's or Sidak's post-hoc test. A Kruskal-Wallis test was used for data that were not normally distributed. For LTP experiments and latency in the Barnes maze, a 2-way repeated measures ANOVA was performed. A Brown-Forsythe one-way ANOVA was used to analyze groups with unequal variance. Significance was defined as $p<0.05$. All error bars are shown as SEM unless otherwise specified.

III: Results

Pathological increases in glutamate drive the excitotoxic synaptic changes and cell death that underlie many neurodegenerative disorders (Wang and Reddy, 2017; Tuo et al., 2022; Verma et al., 2022), including HAND (Haughey et al., 2001 ; Nakanishi et al., 2016; Green et al., 2018). Pharmacological attenuation of excess glutamatergic synaptic activity has shown promise for preventing HIV-associated neurodegenerative processes in neuronal cultures (Kim et al., 2008; Rumbaugh et al., 2012), animal models (Raybuck et al., 2017), and human clinical trials (Zhao et al., 2010). Here we used the GFAP-driven, doxycycline-inducible HIV TAT (iTAT) mouse model of HAND (Kim et al., 2003; Carey et al., 2012) to evaluate the neuroprotective properties of the antiepileptic drug LEV on changes in neurotransmission, synaptic plasticity, and cognitive function induced by TAT expression.

Mice were implanted with osmotic minipumps to administer LEV (150mg/kg/day) or saline (SAL) 14 days before experiments and were then given daily IP injections of DOX (100 mg/kg) for 7 d, starting 1 week after surgery (Fig. 1*A*). One week of daily DOX injections was shown previously to induce robust expression of HIV TAT protein in iTAT mice (Carey et al., 2012). Real-time quantitative reverse transcription PCR was used to confirm expression of TAT mRNA following DOX administration to iTAT mice. TAT mRNA was undetectable in DOX-treated WT mice and SAL-treated iTAT mice.

LEV administration prevents a TAT-induced increase in sEPSC frequency

The state of glutamatergic synaptic transmission can be assessed by measuring the frequency and amplitude of spontaneous excitatory post-synaptic currents

(sEPSCs). Because HAND patients show impairment in cognitive tasks requiring the hippocampus and synapse loss in this region is indicative of cognitive decline in HAND (Moore et al., 2006; Ellis et al., 2007), we recorded sEPSCs in hippocampal slices prepared from iTAT and WT mice. Previous recordings from the medial pre-frontal cortex of iTAT mice found that TAT expression increased sEPSC frequency (Jacobs et al., 2019). Electrophysiological experiments were conducted on hippocampal slices using the whole-cell configuration of the patch-clamp technique. CA1 neurons were clamped at -65 mV, and then 50 µM picrotoxin was added to ACSF perfused continuously through the recording chamber. After allowing the picrotoxin to equilibrate with the slice for 5 min, sEPSCs were recorded for a subsequent 5 min recording period from which amplitude and frequency values were determined. TAT expression increased the sEPSC frequency to 0.96 ± 0.1 Hz, which was significantly higher than the WT frequency of 0.6 ± 0.03 Hz (Fig. 1*B*-*C*). Treatment *in vivo* with LEV for 2 weeks prior to recordings did not significantly affect the sEPSC frequency in WT mice. However, it prevented the increase in frequency observed in the iTAT mice treated with DOX (Fig. 1*B*-*C*). At the time of brain slice preparation, the concentration of LEV in the plasma was 8.3 ± 1.1 µg/mL, which is at the low end of the therapeutic range used for treating seizures in veterinary medicine (12-45 µg/mL, Clinical Pharmacology, Auburn University, vetmed.auburn.edu) and is comparable to the therapeutic range of 10-50 µg/mL for treating human epilepsy (Perucca et al., 2003). LEV was not added to the ACSF perfusing the slice chamber, nor was it added to the cutting or incubation solutions used to prepare the brain slices. Thus, LEV appears to prevent changes in excitatory networks evoked by the presence of HIV-1 TAT.

The sEPSC amplitude was not different among groups (Fig 1*D*), indicating that TAT increases the probability of glutamate release without affecting postsynaptic sensitivity to glutamate. Corresponding cumulative probability graphs for inter-event interval (IEI) and amplitude support this conclusion as shown in Fig. 1*E-F*. Consistent with the increased sEPSC frequency observed in slice recordings from iTAT mice treated with DOX, the IEI was reduced in recordings from these animals (Fig. 1E). The IEI trended higher in recordings from WT animals in the presence of LEV relative to SAL, although this difference did not reach statistical significance (p=0.06). Overall, LEV protected glutamatergic networks from increased activity without significantly affecting synaptic transmission under basal (WT) conditions.

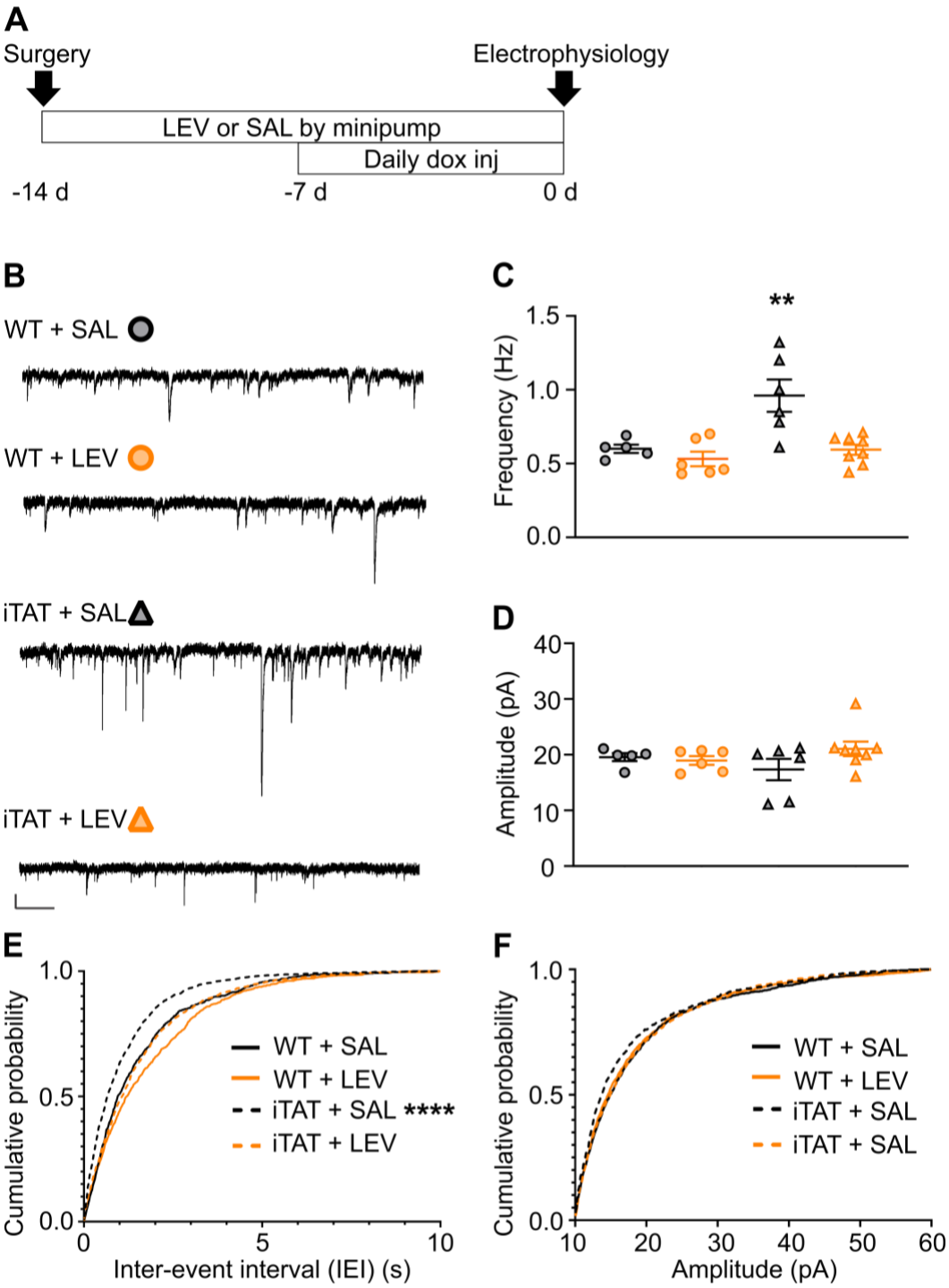

Fig 1. LEV administration prevented a TAT-induced increase in sEPSC frequency. **A**, Experimental timeline for electrophysiology experiments. Osmotic minipumps containing LEV (150 mg/kg/d) or SAL were implanted (surgery) 2 weeks prior to electrophysiological recordings. All mice received daily IP injections (0.3 mL/30 mg) of DOX *(100 mg/kg* dissolved in 0.9% SAL*)* for one week, were then euthanized, and hippocampal slices prepared for electrophysiological recordings as described in

Methods. **B**, Representative sEPSC traces from each experimental group are shown (scale bar: 5 pA/ 1 s). Legend applies to **B-D**. **C**, Bar graph shows sEPSC frequency determined from brain slice recordings from each of the treatment groups shown in **B**. n = 5-8 recordings for each condition. 2-way ANOVA: genotype $F_{(1,21)} = 11.2$, $p<0.01$; drug treatment $F_{(1,21)} = 11.8$, $p<0.01$; interaction $F_{(1,21)} = 5.6$, $p<0.05$; Tukey's post-hoc test: ** $p < 0.01$ compared to iTAT + LEV and WT + SAL. **D**, Bar graph shows sEPSC amplitude determined from brain slice recordings from each of the treatment groups shown in **B**. n = 5-8 recordings for each condition. No significant changes were detected. 2-way ANOVA: genotype $F_{(1,21)} = 0.0002$, $p=0.97$; drug treatment $F_{(1,21)} = 1.3$, $p=0.26$; interaction $F_{(1,21)} = 2.5$, $p = 0.13$;. **E**, Inter-event interval (IEI) cumulative probability is shown for each experimental group. iTAT + DOX + SAL mice had a significantly shorter IEI (Kruskal-Wallis: $H = 55.2$; $p < 0.0001$; Dunn's post-hoc test: **** $p < 0.0001$ compared to iTAT + LEV, WT + SAL, and WT + LEV.). **F**, Amplitude cumulative probability is shown for each experimental group. No significant changes were detected (Kruskal-Wallis with Dunn's post-hoc test: $H = 5.6$; $p = 0.13$).

LEV requires prolonged incubation to prevent the TAT-induced increase in sEPSC frequency

Having established that chronic LEV administration *in vivo* decreased sEPSC frequency in iTAT mice, we next determined if acute application of LEV on brain slices from mice without minipumps would also be effective. Many drugs applied to the slice by perfusion, including the picrotoxin used in this study, penetrate the slice in 5 min or less. After application of LEV (4 µg/mL) by perfusion at the same time as picrotoxin and following equilibration with the slice for 5-10 min (Fig. 2*A*-*B*; iTAT + acute LEV), the iTAT sEPSC frequency was 0.89 ± 0.07 Hz. This value is comparable to recordings from SAL-treated TAT-expressing mice (iTAT + SAL (Fig. 1*D*); $t_{(8)}$=0.51, p=0.62, two-tailed Student's T-test), indicating that there was no acute effect of LEV on sEPSC frequency.

However, when iTAT slices were allowed to incubate with 4 µg/mL LEV for 1-4 h in a holding chamber prior to recording and LEV was continuously perfused through the slice chamber during the recording, sEPSC frequency was reduced significantly from 0.89 ± 0.07 Hz in iTAT mice treated acutely with LEV (iTAT + acute LEV) to 0.50 ± 0.05 Hz (iTAT + 1-4 h LEV) (Fig 2*A*-*B*). The sEPSC frequency recorded from slices from WT mice treated with LEV for 1-4 h was 0.52 ± 0.08 Hz (Fig 2*A*-*B*). This value is comparable to sEPSC frequencies recorded in slices from WT mice treated with SAL *in vivo* (Fig. 1*D* WT + SAL; $t_{(7)}$=1.1, p=0.31, two-tailed Student's T-test) and WT mice treated with LEV *in vivo* (Fig. 1*D* WT + LEV; $t_{(8)}$=0.16, p=0.87, two-tailed Student's T-test).

IEI cumulative probability graphs corresponding to the acute and 1-4 h treatment groups are consistent with acute LEV failing to reverse the TAT-induced increase in sEPSC frequency whereas 1-4 h treatment returned sEPSC frequency to control levels (Fig. 2*D*). The sEPSC amplitude was not significantly different among groups treated with SAL, acute LEV, or LEV for 1-4 h (Fig 2*C*). This is also apparent in the amplitude cumulative probabilities (overlaid graphs shown in Fig. 2*E*). In summary, application of LEV *in vitro* reversed the increase in sEPSC frequency established *in vivo*. However, this reversal required approximately 1 h to become apparent.

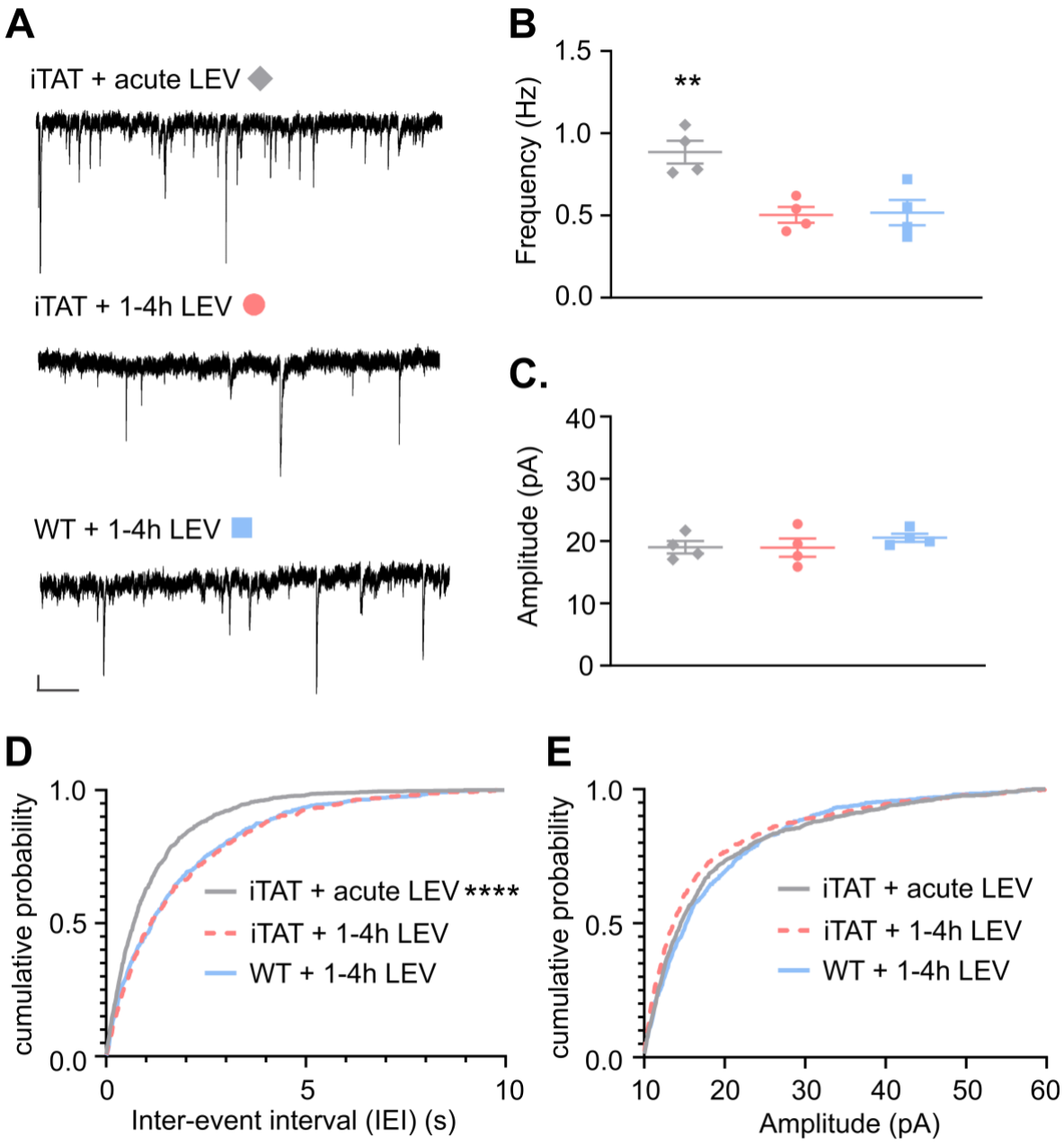

Fig 2. 1-4 h incubation with LEV, but not acute application, prevented TAT-induced increase in sEPSC frequency. A, representative sEPSC traces from each experimental group are shown (scale bar: 5 pA/1 s). Legend applies for **A-C**. **B**, bar graph shows sEPSC frequencies for groups shown in **A**. The sEPSC frequency following acute application of LEV (5-10 min) to slices from iTAT mice (iTAT + acute LEV) was significantly elevated relative to WT and iTAT mice incubated with LEV for 1-4 h. n = 4 recordings for each condition. One-way ANOVA: $F_{(2,9)} = 10.71$, P = 0.0042; Tukey's post-hoc test: **p < 0.01 compared to iTAT + 1-4 h LEV and WT +1-4 h LEV. **C,** Bar graph shows sEPSC amplitudes for groups shown in **A**. No significant changes were detected. n = 4 recordings for each condition. One-way ANOVA: $F_{(2,9)}$= 0.67,

p=0.54. **D,** IEI cumulative probability plot shows each experimental group described in **A**. Note that iTAT + acute LEV mice had an increased probability of shorter event intervals (Kruskal-Wallis: $H = 97.95$, $p < 0.0001$; Dunn's post-hoc test: *** $p < 0.0001$ compared to iTAT + 1-4h LEV and WT + 1-4h LEV.). **E,** Amplitude cumulative probability shown for each experimental group described in A. No significant changes were detected (Kruskal-Wallis with Dunn's post-hoc test: $H = 3.4$; $p = 0.18$).

TAT expression does not change sIPSC frequency or amplitude

The balance between excitatory and inhibitory signaling is disrupted in HAND (Nass et al., 2020), which can increase susceptibility to excitotoxic damage and synaptic injury. Thus, we tested whether TAT expression altered spontaneous inhibitory post-synaptic currents (sIPSCs; Fig 3*A*). APV (50 μM) and DNQX (10 μM) were present in all recordings. sIPSC frequencies and amplitudes recorded from WT animals (WT +DOX) were comparable to values previously reported for hippocampal slice (Jones and Baraban, 2009; Fusilier et al., 2021). Neither sIPSC frequency (WT+DOX: 9.1 ± 0.1 Hz; iTAT+DOX: 8.4 ± 0.8 Hz) nor amplitude (WT+DOX: 45 ± 6 pA; iTAT + DOX: 42 ± 5 pA) were significantly different in recordings from WT relative to iTAT mice (frequency: Welch's t-test, $t_{(4.2)}$= 0.89, p=0.42; amplitude: Student's t-test, $t_{(8)}$=0.4, p=0.7). Cumulative probability plots for frequency and amplitude are shown in Fig 3*B* and 3*C*, respectively. TAT expression did not affect sIPSCs, suggesting that the TAT-induced increase in excitatory signaling (Figs. 1 and 2) is not accompanied by a compensatory increase in inhibitory signaling. As such, LEV was not tested in this assay.

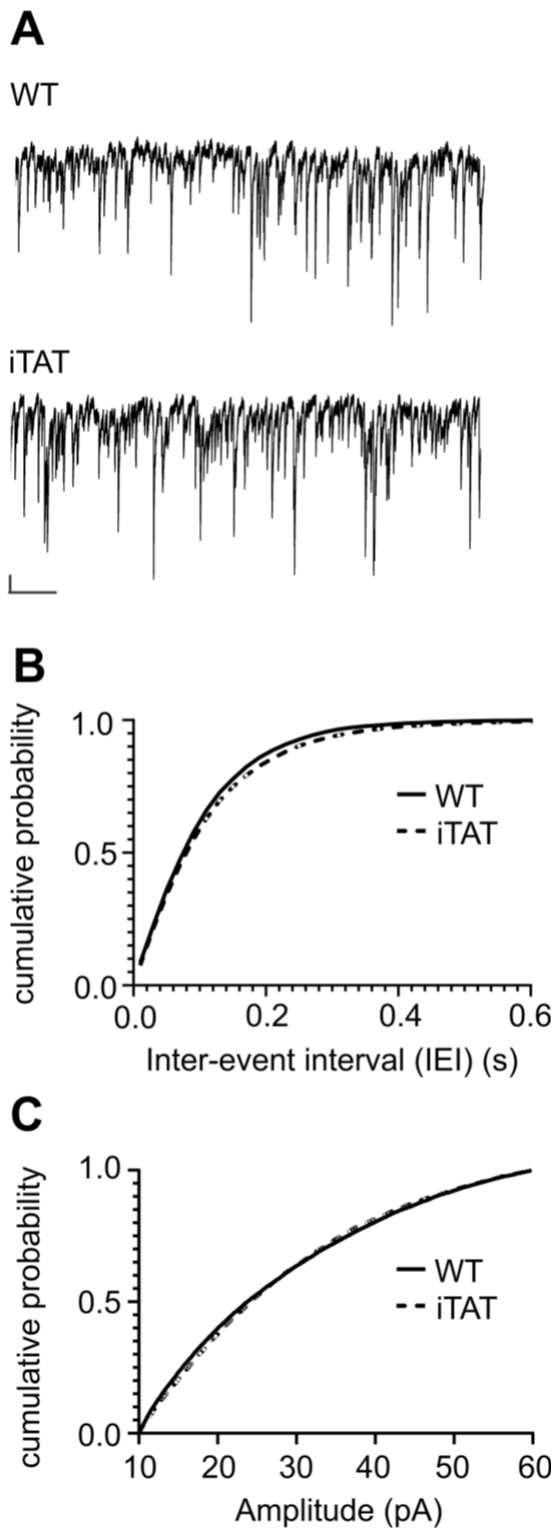

Fig 3. sIPSC frequency and amplitude were unchanged by TAT expression. A, representative traces show the effect of TAT expression on sIPSCs (scale bar: 5 pA/1 s). **B**, IEI cumulative probability shown for each experimental group in s. No significant changes were detected (Kolmogorov-Smirnov test D = 0.03, p=0.99). **C,** Amplitude cumulative probability shown for each experimental group in pA. No significant changes were detected (Kolmogorov-Smirnov test D = 0.02, p=0.99). n = 5 recordings for each condition.

LEV prevents TAT-induced impairment of long-term potentiation (LTP)

Long-term potentiation (LTP) is a robust assay for examining synaptic plasticity *in vitro* that underlies learning and memory *in vivo* (Nicoll, 2017). iTAT mice fed DOX in their chow for one month were shown to have impaired hippocampal LTP (Fitting et al., 2013). We replicated this finding using the IP DOX protocol used in this study prior to examining the potential protective properties of LEV treatment. Field excitatory postsynaptic potentials (fEPSPs) were recorded in hippocampal slices from stratum radiatum and evoked by a concentric bipolar electrode stimulating Schaffer collaterals upstream of the recording electrode. LTP was significantly impaired in iTAT mice. In recordings from WT mice, the fEPSP slope, an index of synaptic strength, increased to 239 ± 35% of baseline following a brief high frequency stimulus (100 Hz, 1s). In contrast, in slice recordings from iTAT mice, the fEPSP slope increased to 124 ± 4% of baseline, a significantly smaller change relative to WT (2-way repeated measures ANOVA: Time $F_{(1.8, 16.2)} = 8.5$, p=0.0037; genotype $F_{(1, 9)} = 29.7$, p = 0.0004; time x genotype interaction $F_{(9, 99)} = 10.8$, $p < 0.0001$; data not shown). We next examined whether this TAT-induced impairment in synaptic plasticity could be prevented with two-week administration of LEV using osmotic minipumps (Fig. 1*A*). LEV treatment prevented LTP impairment in TAT-expressing mice; following HFS, the fEPSP slope in slices from iTAT + LEV mice increased to 264 ± 23 % of baseline (Fig 4*A*). iTAT mice administered SAL by minipump exhibited impaired LTP, reaching only 117 ± 10 % of baseline. Thus, LTP in iTAT mice treated with LEV was significantly greater than animals treated with SAL. LTP in WT animals treated with LEV was 199 ± 7% of baseline, which was not significantly different from the 242 ± 26% increase observed in

animals that received SAL (Fig. 4*B*). Results for animals receiving LEV or SAL by minipump are summarized in Fig. 4*C* in which the fEPSP slope relative to baseline is shown 50 min after high-frequency stimulation. LEV administration prevented the impaired synaptic plasticity that results from the expression of TAT.

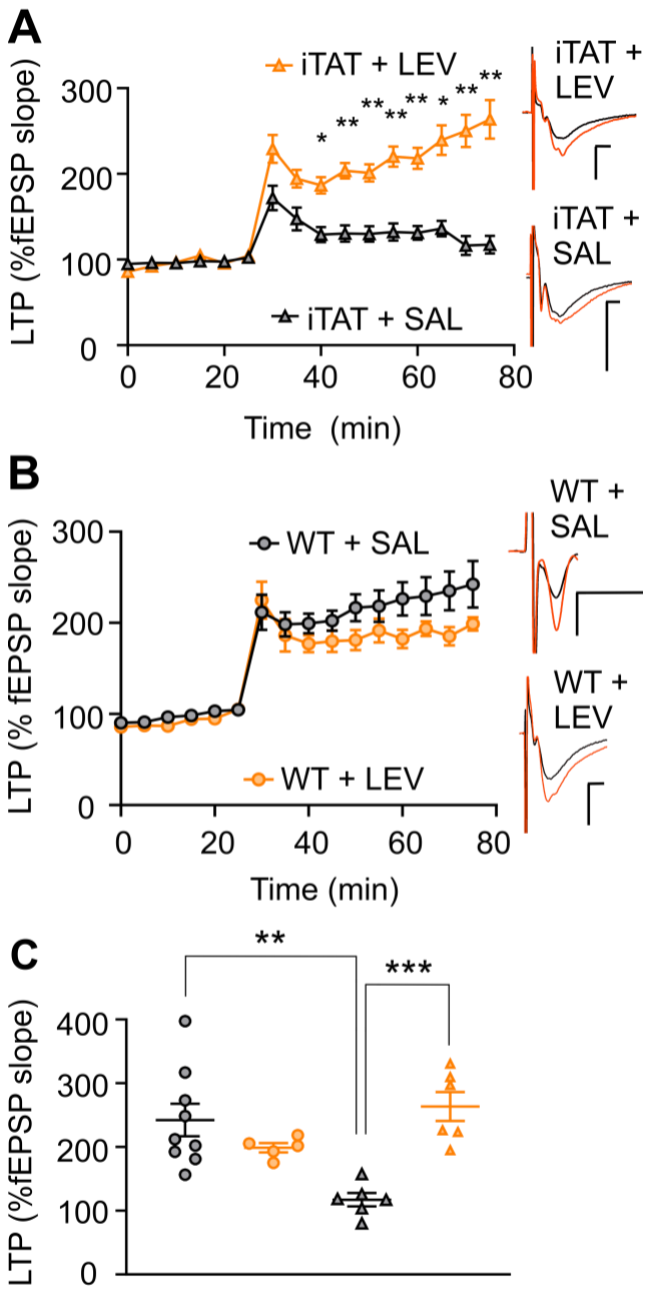

Fig 4. LEV prevented TAT-induced impairment of LTP. A-C, fEPSPs were recorded in hippocampal slices from stratum radiatum and evoked every 30 s by a concentric bipolar electrode stimulating Schaffer collaterals. LTP was evoked by high frequency stimulation (HFS; 100 Hz, 1s) after 30 minutes of baseline recording. Plots show mean fEPSP slopes normalized to the average of baseline sweeps during the last 10 min immediately preceding HFS. **A**, Mice expressing TAT treated with SAL (iTAT + SAL) had an impaired LTP response, which was prevented by two-week LEV administration (iTAT + LEV). LEV and SAL were administered by osmotic minipump as described in methods. 2-way repeated measures ANOVA: time $F_{(3.2, 32.0)} = 48.6$, $p < 0.0001$; drug treatment $F_{(1, 10)} = 35.4$, $p = 0.0001$; interaction $F_{(15, 150)} = 18.1$, $p ,< 0.0001$. Sidak's post-hoc test: *$p < 0.05$, **$p < 0.01$. *Insets* show representative fEPSP traces from baseline (black; mean of 10 sweeps preceding HFS) and 50 min after HFS (red; mean of 10 sweeps). Scale bars = 5 ms/0.5 mV). **B,** Two-week LEV administration by minipump to WT mice (WT + LEV) did not significantly suppress LTP relative to SAL control (WT + SAL). 2-way repeated measures ANOVA: time $F_{(2.2, 26.6)} = 45.33$, $p < 0.0001$; drug treatment $F_{(1, 12)} = 2.15$, $p = 0.17$; interaction $F_{(15, 180)} = 1.26$, $p = 0.23$. *Insets* show representative fEPSP traces from baseline (black; mean of 10 sweeps preceding HFS) and 50 min after HFS (red; mean of 10 sweeps). Scale bars = 5 ms/0.5 mV). **C,** Bar graph summarizes changes in LTP produced by TAT expression and LEV administration. The change in fEPSP slope (% of baseline) 50 min after HFS is shown for n=5-9. (2-way ANOVA: genotype $F_{(1,22)} = 1.87$, $p=0.19$; drug treatment $F_{(1, 22)} = 5.4$, $p = 0.03$; interaction $F_{(1,22)} = 18.5$, $p = 0.0003$. Tukey's post-hoc test: ** $p < 0.01$ compared to WT + SAL, ***$p<0.001$ compared to iTAT + LEV.

TAT expression increases seizure susceptibility without causing overt epilepsy

Alzheimer's disease-model mice were previously found to have subclinical epileptiform spikes in hippocampal EEG recordings; the frequency of these abnormal spikes was attenuated by LEV (Sanchez et al., 2012). Since the electrophysiological data described here shows that TAT expression increased excitatory signaling that was sensitive to LEV, we wanted to determine whether iTAT + DOX mice exhibited abnormal spiking activity. Analysis of EEG recordings from iTAT mice treated with DOX for 7 d failed to detect overt seizures (7 days of continuous video EEG monitoring) or overt epileptiform spikes (n=8 mice, iTAT + DOX). As changes in alpha and theta power have been associated with several neurological disorders (Klimesch 1999), spectral power in the recorded signals, from both dorsal and ventral hippocampus, was also analyzed for the last 24h after 7 d of DOX or SAL administration (Fig. 5*A,B*). While there was a trend towards increased power in higher frequency bands in the dorsal hippocampus in iTAT + DOX mice, no significant differences were seen at either location. Together, these data suggest that iTAT+DOX mice are not overtly epileptic (that is, they do not display spontaneous seizures) at this stage of disease in this model, and therefore that LEV is unlikely to be having its impact via suppression of epileptiform activity.

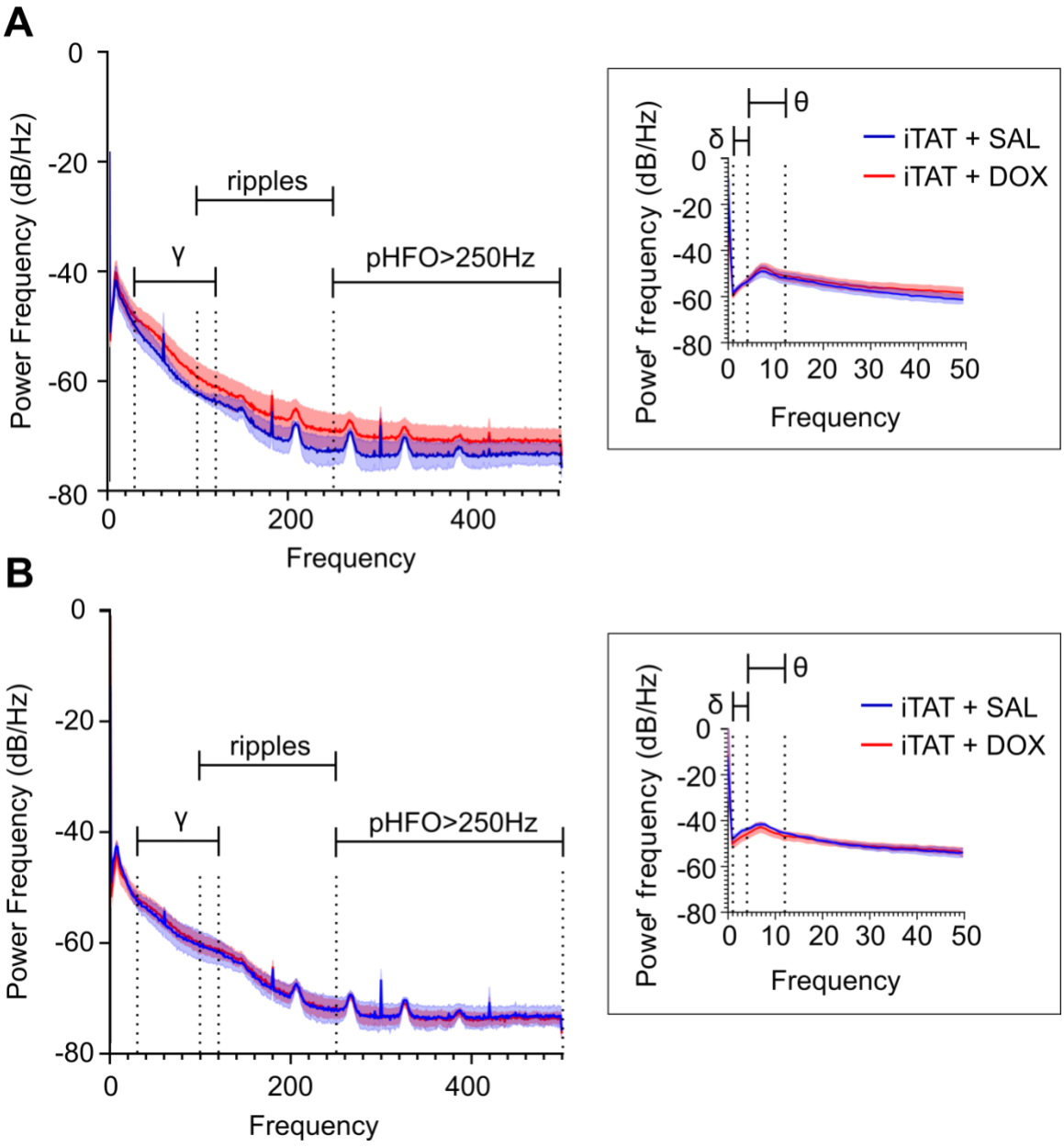

Fig 5. TAT expression did not change hippocampal LFP power spectral density. **A-B**, Power spectral density (PSD) plots of recordings using hippocampal depth electrodes from iTAT + SAL (blue) and iTAT + DOX (red) mice (24 h analysis after 7 d daily DOX or SAL administration) reveal no significant changes in power (iTAT + SAL vs iTAT + DOX Channel A total power: Student's t-test, $t_{(5)} = 0.24$; $p = 0.82$; iTAT + SAL vs iTAT + DOX Channel B total power: Student's t-test, $t_{(5)} = 1.16$; $p = 0.30$). (**A**, dorsal hippocampal recording location; **B**, ventral hippocampal recording location). Insets show PSD plotted on an expanded low frequency scale. Data are plotted as mean (dark line) ± SEM (shaded section).

However, hyperexcitability and an associated increased seizure risk may still be present. Indeed, there is evidence that systemic TAT may increase hyperexcitability, seen as an increased seizure severity to KA (Zucchini 2013). We therefore wanted to determine whether selective expression of TAT in the CNS would alter seizure threshold or severity. We induced seizures with KA in iTAT + DOX and iTAT + SAL mice at 40 mg/kg. At this dose, 100% of iTAT + DOX mice reached stage 3 compared to 50% of iTAT + SAL mice (Fig 6*A*). In mice treated with LEV, (200 mg/kg, I.P.) all but one mouse failed to develop stage 3 seizures (n=12 mice, Fig 6*A*). Mice were assigned a behavioral score every min for 2 h following KA administration (shown in 5 min bins, Fig 6*B*). Summing Racine scores across time captures both the severity and duration of evoked seizures as reflected in the cumulative scores shown in Figure 6*C*. iTAT mice receiving DOX exhibited significantly greater cumulative scores relative to non-induced, SAL-treated mice. Furthermore, IP administration of LEV 1 h prior to KA injection markedly attenuated KA-induced seizures in iTAT mice. To monitor electrographic seizure events, a separate cohort of mice were implanted with EEG depth electrodes, administered 30 mg/kg KA SQ, and observed for 2h. Representative depth EEG traces in Fig. 6*D* show the first electrographic seizure event in an iTAT + DOX mouse occurring at 21 minutes following KA injection, compared to 31 minutes in an iTAT + SAL mouse (boxes indicate electrographic seizures expanded in inset). At this dose, the iTAT + DOX + KA mice briefly reached stage 3 on the Racine behavioral scale approximately 55-60 min after KA injection; iTAT + SAL + KA mice did not pass stage 1 (data not shown). Together, these results indicate that TAT expression increases seizure susceptibility, indicating

hyperexcitability. LEV treatment effectively prevented the propensity to KA-evoked seizures, suggesting LEV-mediated reversal of hyperexcitability in these animals.

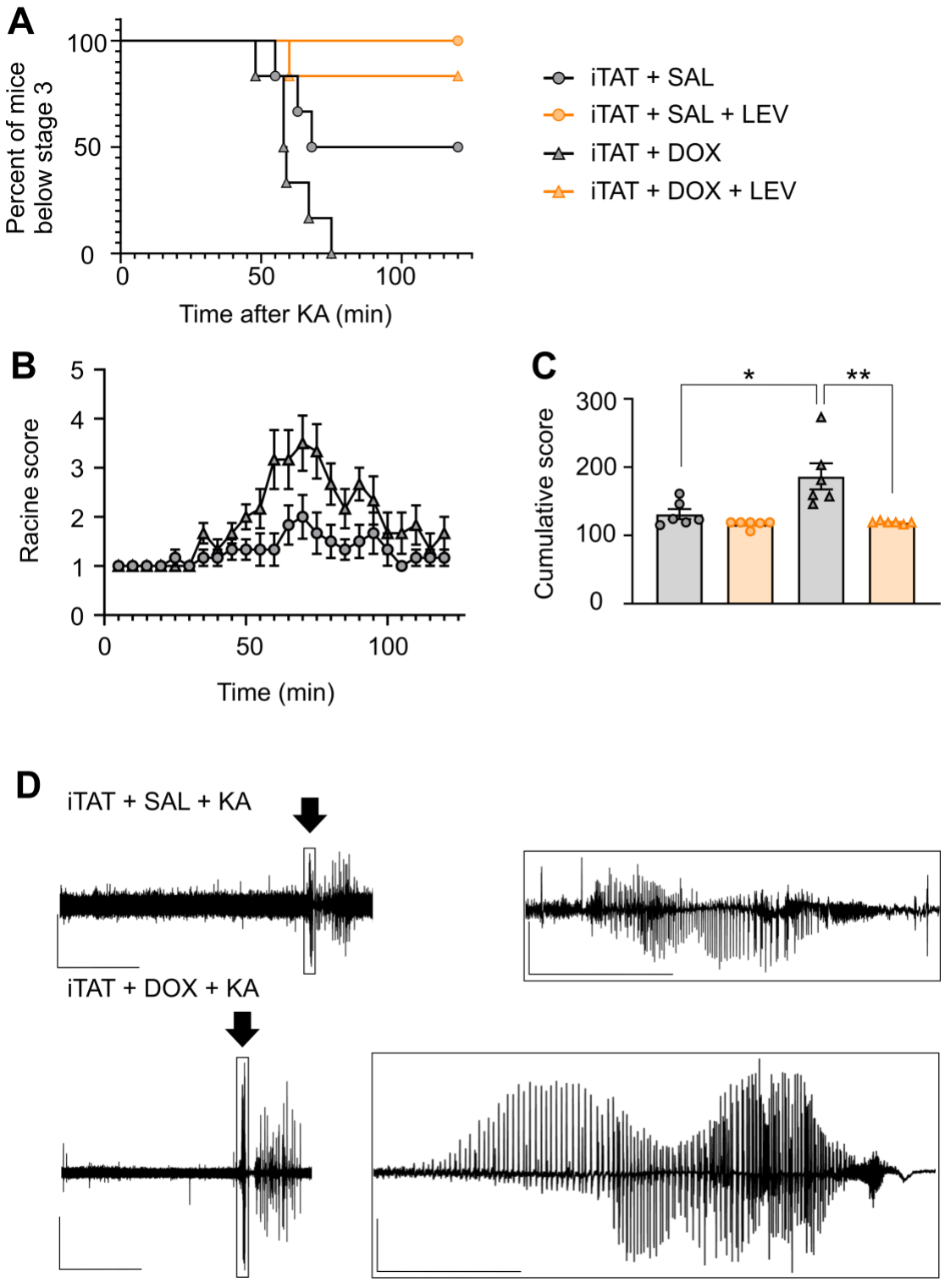

Fig 6. LEV reduces TAT-induced susceptibility to KA-induced seizures. A, Survival graph following 40 mg/kg KA SQ shows percent of mice reaching Racine stage 3 during 2 h observation period ($X^2(3, n=24) = 18.4$, $p < 0.001$, Log rank (Mantel-Cox) test for all groups; ($X^2(1, n=12) = 8.7$, $p=0.0031$, Log rank (Mantel-Cox) for iTAT + DOX compared

to iTAT + DOX + LEV). Mice were administered SAL or LEV IP (200 mg/kg) 1 h prior to KA injection. Legend applies for **A-C**. **B**, Seizure scores of iTAT + SAL and iTAT + DOX mice following KA administration, graphed in 5 min bins. Scores were validated by blinded observer. **C**, Cumulative Racine score for each group described in **A** over entire 2-hour observation period (Brown-Forsythe ANOVA with Dunnett's post-test on log-transformed data, * = $p < 0.05$; ** = $p < 0.01$). **D**, Representative EEG traces from iTAT + SAL and iTAT + DOX mice during the first hour following KA (30mg/kg) administration (iTAT + SAL total time shown = 42 mins; iTAT + DOX total time shown 30 mins). Boxed area represents first seizure (arrow), which is enlarged to the right. Scale bar: 0.5 V / 10 min (0.5V / 10 s for expanded trace).

LEV protects against TAT-induced learning impairments

The same groups used in previous experiments (iTAT and WT mice implanted with SAL or LEV minipumps) were used for behavioral testing. A series of two behavioral tests began 1-3 d after the last DOX injection, starting with novel object recognition (NOR). NOR is a well-established behavioral assay that is used to assess memory, as mice are more likely to explore novel objects than familiar ones (Antunes and Biala, 2012). The time spent with the novel object is ratioed to total object interaction time to calculate a recognition index (Hammond et al., 2004; Taglialatela et al., 2009). NOR short-term memory was first tested 3-hours after familiarization with objects resulting in recognition indices for WT + SAL, WT + LEV, iTAT + SAL and iTAT + LEV were 0.51 ± 0.04, 0.53 ± 0.04, 0.55 ± 0.05, and 0.59 ± 0.04, respectively. A two-way ANOVA showed no significant effects of genotype, treatment, or interaction (genotype $F_{(1,39)} = 1.4$, p=0.24; treatment $F_{(1, 39)} = 0.61$, p = 0.44; interaction $F_{(1,39)} = 0.076$, p = 0.78). NOR long-term memory was subsequently tested 24-hours later (Fig. 7A); a two-way ANOVA showed significant effects of genotype, but not treatment or interaction. Mice expressing TAT trended towards impairment in the 24-hour NOR test (p=0.14), although none of the posttest comparisons were significant. Locomotor activity was also measured during NOR habituation. There were no significant differences in distance traveled during habituation among the 4 groups. The distance traveled for WT + SAL, WT + LEV, iTAT + SAL and ITAT + LEV were 25.3 ± 1.6 m, 23.5 ± 1.7 m, 23.5 ± 1.3 m, and 23.2 ± 1.4 m, respectively. A two-way ANOVA found no effect of genotype ($F_{(1,39)} =0.45$; p=0.51), drug treatment ($F_{(1,39)} =0.50$; p=0.48), or interaction ($F_{(1,39)} =0.24$; p=0.63).

Barnes maze training began 48 h following completion of NOR. The Barnes maze is a reliable measurement of spatial learning and memory in rodents, where mice are motivated to locate an escape box due to their instinct to avoid open, well-lit environments in favor of dark ones (Pitts, 2018). Configuration of the goal hole and goal quadrant are shown in Fig 7*B*. LEV did not alter latency to escape in WT (Fig 7*C*), but significantly reduced the latency for iTAT mice to find the goal hole (Fig 7*D*). iTAT + LEV mice had a decreased latency to escape on Days 1 and 2 compared iTAT + SAL. However, this difference was no longer significant on days 3 and 4 as the training decreased the time all groups of mice required to find the goal hole.

The latency for mice to find the goal hole In the Barnes maze reflects errors associated with learning the task. Total, reference, and working errors were measured during acquisition; reference errors represent the first time a mouse enters an incorrect hole, while working errors represent any subsequent time a mouse enters an incorrect hole. The iTAT + SAL group made more total, reference, and working errors than the WT + SAL group (Fig. 7*E*). LEV attenuated this increase in all 3 types of errors observed in mice expressing TAT (Fig 7*E*). LEV had no effect on the number of errors made by WT mice.

In contrast to the reduced latency to find the goal and the reduction in the number of errors produced by treatment of TAT expressing mice with LEV, both iTAT + SAL and iTAT + LEV groups were impaired in the probe test compared to WT + SAL (Fig 7*F*), suggesting that LEV may not fully rescue long-term spatial memory. The probe test is a single trial in which time spent in the goal quadrant is measured. Because the latency data shown in Figures 7*C* and *D* are the means of 4 trials on each day, we

examined the escape latency for the first trial on each day in iTAT mice in the absence and presence of LEV. 2-way repeated measures ANOVA revealed effects of treatment ($F_{(1,23)}$ = 13.84; p = 0.0011), time ($F_{(2.38,54.82)}$ = 36.95; p <0.0001), and interaction ($F_{(3,69)}$ = 3.85; p=0.013). The iTAT + SAL mice had significantly longer latencies relative to the iTAT + LEV group on day 2 (p<0.01) and trending on day 3 (p=0.078; Sidak's post-hoc test). The analysis of the first trial of the day during training showed similar results to latencies that were averaged over the four trials. Thus, the discrepancy between the effects of LEV on the latency of TAT-expressing mice to escape versus the lack of effect of LEV on their performance in the probe test cannot be explained by within-day learning.

No significant differences were detected for latency to escape during the reversal test (data not shown). However, LEV reduced the number of working errors in the reversal test in mice expressing TAT (2-way ANOVA: genotype $F_{(1,47)}$ = 5.78, p=0.02; treatment $F_{(1, 47)}$ = 4.3, p=0.044; interaction $F_{(1,47)}$ = 3.67, p=0.062, Tukey's post-hoc test p=0.037 for iTAT + SAL compared to iTAT + LEV). Total errors in the reversal test were increased by TAT expression but the attenuation by LEV did not reach statistical significance (2-way ANOVA: genotype $F_{(1,47)}$ = 6.67, p=0.013; treatment $F_{(1, 47)}$ = 3.95, p = 0.053; interaction $F_{(1,47)}$ = 2.15, p = 0.15).

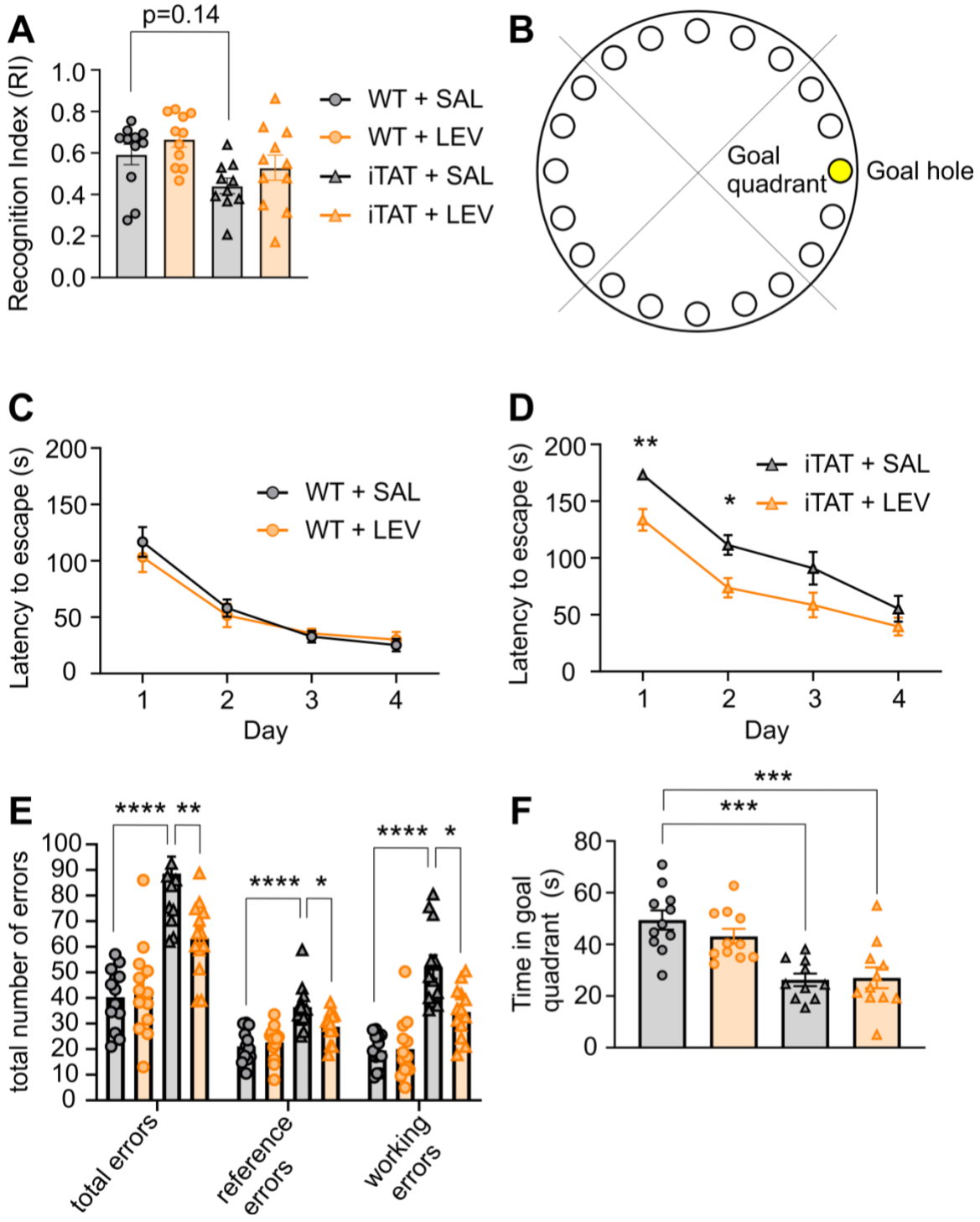

Fig 7. LEV prevents some TAT-induced learning impairments in the Barnes maze. **A,** NOR testing 24 h after familiarization found an effect of TAT expression; a two-way ANOVA showed significant effects of genotype, but not treatment or interaction (genotype $F_{(1,39)} = 9.1$, p=0.0043; treatment $F_{(1, 39)} = 2.8$, p = 0.099; interaction $F_{(1,39)} = 0.022$, p = 0.88). **B**, scheme depicting configuration of the Barnes maze, goal hole location, and goal quadrant. **C-D,** latency to put all four paws in escape hole, displayed

as an average of 4 trials per day. **C**, LEV did not significantly affect the latency for WT mice to find the goal (2-way repeated measures ANOVA; time $F_{(2.02,48.38)}$= 46.21, p<0.0001; treatment $F_{(1,24)}$=0.13, p=0.72; interaction $F_{(3,72)}$=0.60, p=0.61). **D**, iTAT + SAL mice took significantly more time to escape than iTAT + LEV (2-way repeated measures ANOVA: time $F_{(2.31,53.11)}$=81.8, p<0.0001; treatment $F_{(1,23)}$=8.43, p=0.008; interaction $F_{(3,69)}$=1.2, p=0.32; *, p<0.05, **, p<0.01; Sidak's post-hoc test), **E,** Bar graphs show total, reference, and working errors over the 4-day acquisition period. Each type of error was analyzed by 2-way ANOVA for the effects of TAT expression (genotype) and LEV (treatment). Analysis of total errors revealed effects of genotype ($F_{(1,47)}$ = 51.31, p<0.0001) and treatment ($F_{(1,47)}$ = 5.62, p=0.022) with a significant genotype x treatment interaction ($F_{(1,47)}$ = 8.42, p=0.0056). Analysis of reference errors revealed effects of genotype ($F_{(1,47)}$ = 30.12, p<0.0001), but not treatment alone ($F_{(1,47)}$ = 2.46, p=0.12) with a significant genotype x treatment interaction ($F_{(1,47)}$ = 5.51, p=0.023). Analysis of working errors revealed effects of genotype ($F_{(1,47)}$ = 53.96, p<0.0001) and treatment ($F_{(1,47)}$ = 7.08, p=0.011) with a significant genotype x treatment interaction ($F_{(1,47)}$ = 7.94, p=0.0071). *, p < 0.05; **, p< 0.01, ***, p<0.001, ****, p<0.0001 Tukey's posttest. **F,** Time spent in goal quadrant during the probe test. 2-way ANOVA revealed an effect of genotype ($F_{(1,39)}$ = 33.82, p<0.0001) but no significant effect of treatment ($F_{(1,39)}$ = 0.68, p=0.41) and there was not a significant interaction ($F_{(1,39)}$ = 1.13, p=0.29).

The type of strategy used during acquisition of the Barnes maze task was determined using Barnes maze unbiased strategy software (BUNS) (Illouz et al., 2016). There are multiple search strategies that mice employ to locate the escape hole during the Barnes maze assay; each is assigned a number from 0-1 that represents their effectiveness and amount of spatial memory required. Random is assigned 0; serial is a slightly better strategy and is assigned 0.25; focused search involves a search in the quadrant surrounding the escape hole, with long correction being similar, but with a smaller radius – both are assigned 0.5; corrected involves a deviation one hole away from the escape hole and is assigned 0.75; direct is a path directly to the escape hole and is completely spatially-dependent, so is assigned a score of 1. Representative traces of the three major strategies (random, serial, and direct) are shown in Fig 8*A*. BUNS analysis determined that iTAT + LEV mice had a significantly better cognitive score than iTAT + SAL mice over the four training days (Fig 8*B*). The distribution of strategy use for each group is shown in Fig 8*C*. The increased latency to escape, coupled with the increased number of total, reference, and working memory errors, as well as decreased cognitive score, are consistent with TAT expression impairing learning in the Barnes maze; LEV reduced this impairment.

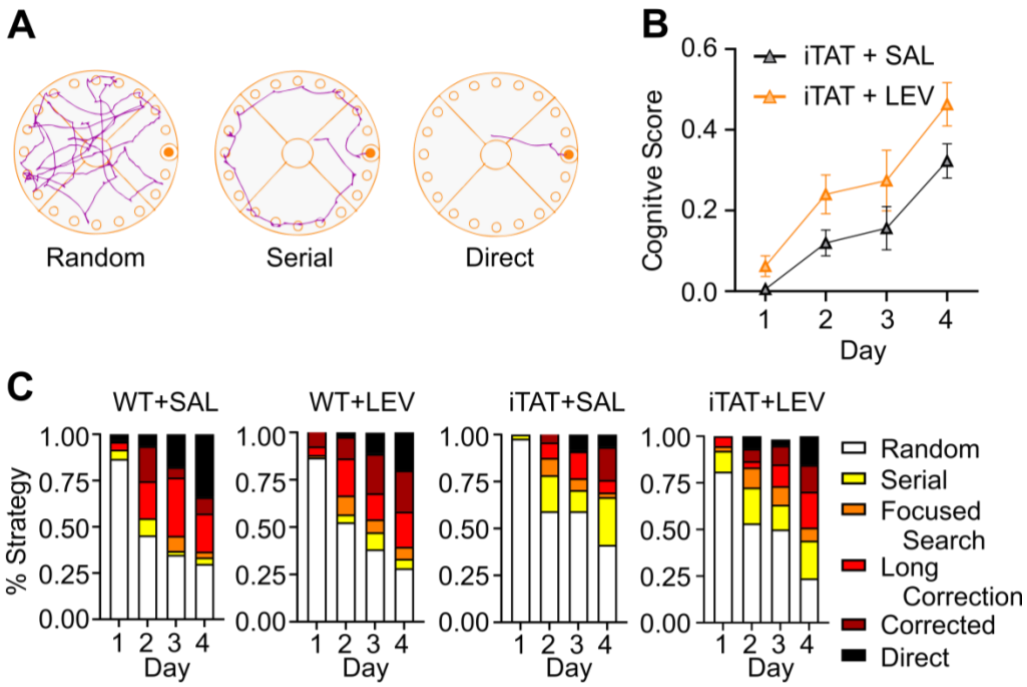

Figure 8. LEV increases cognitive score in TAT-expressing mice performing the Barnes maze assay. A, representative track plots of the show random (WT + SAL mouse id#4), serial (iTAT + LEV mouse id#1964), and direct (iTAT + LEV mouse id#1966) search strategies with the goal hole placed in the same location shown in Fig. 7*B* (solid circle). **B,** cognitive scores calculated from search strategy as described in Methods. A score of 1 is awarded for a direct strategy and 0 for a random search. iTAT mice treated with LEV had a significantly better cognitive score over the acquisition period (2-way repeated measures ANOVA: time $F_{(2.5,57.02)} = 26.71$; $p < 0.0001$; treatment $F_{(1,23)} = 5.93$; $p < 0.05$; interaction $F_{(3,69)} = 0.39$; $p=0.76$). Post-hoc analysis did not reveal significance on particular days (day 1: p=0.17; day 2: p=0.18; day 3: p=0.62; day 4: p=0.2; Sidak's post-hoc analysis). **C,** search strategy types and percent usage, stratified by genotype and treatment group.

IV: Discussion

HAND is a debilitating neurological disorder that currently has no treatment. The HIV protein TAT is thought to be a major contributor to HAND neuropathology and its transgenic expression in the CNS of mice recapitulates cognitive deficits seen in HAND. Here, for the first time, we showed that an antiepileptic drug, LEV, protected from TAT-induced synaptic, network, and behavioral impairments. TAT increased sEPSC frequency, impaired LTP, reduced seizure threshold, and impaired learning acquisition in the Barnes maze; these deficits were prevented by treatment with LEV.

Because neurodegeneration in HAND (Rempel and Pulliam, 2005; Moore et al., 2006; Ru and Tang, 2017; Li et al., 2022) bears some resemblance to AD, our interest in LEV was prompted by its ability to reverse neurological and behavioral deficits in an AD-model mouse (Sanchez et al., 2012). We found that LEV prevented cognitive deficits in iTAT mice, suggesting a common underlying mechanism of neurodegeneration that is exacerbated by excess excitatory synaptic activity. Indeed, there is growing evidence that LEV might afford protection in other neurodegenerative diseases. An ongoing clinical trial testing whether LEV can rescue mild neurocognitive impairment in Parkinson's disease explores this possibility (Dissanayaka et al., 2023). In both Huntington's disease and multiple sclerosis, LEV alleviated involuntary movements (de Tommaso et al., 2005) and tremors (Hawker et al., 2003; Solaro et al., 2020), while cognition was either unchanged or untested. The potentiation of glutamatergic synaptic transmission by inflammatory conditions that accompany neurodegenerative and seizure disorders could explain LEV's broad spectrum of activity (Galic et al., 2012; Mishra et al., 2012; Mediouni et al., 2015a). LEV is the only AED that improves

cognition in Parkinson's disease (Belete et al., 2023), similar to its unique ability to afford neuroprotection in AD models (Sanchez et al., 2012).

The neuroprotective properties of LEV may stem from its unique mechanism of anti-seizure action. LEV decreases neurotransmitter release by acting on synaptic vesicle protein SV2A (Steinhoff and Staack, 2019). SV2A regulates readily releasable pools and influences vesicle priming (Wu et al., 2023). Whether LEV supports or inhibits SV2A is unclear, but ultimately, the interaction of LEV with SV2A decreases glutamate release (Bradberry and Chapman, 2022). This mechanism is consistent with attenuation of the TAT-induced increase in sEPSC frequency observed in this study (Fig. 1). An action on vesicle trafficking might also explain the slow (> 1 h) onset of action in *in vitro* experiments (Fig. 2), although the delayed effects could also result from a slow reversal of network changes induced by TAT. Expression of TAT produces dendritic pruning (Kim et al., 2003) and synapse loss (McLane et al., 2022); TAT-induced loss of synapses *in vitro* is reversible (Shin et al., 2012), consistent with a form of network downscaling (Green et al., 2018). Attenuation of vesicle priming by LEV might create a use-dependent effect that would explain relative sparing of sEPSC frequency in recordings from WT animals (Fig. 1). In addition to its interaction with SV2A, LEV's anti-inflammatory, calcium-channel-blocking, and antioxidant properties (Contreras-García et al., 2022) could contribute to neuroprotection, although LEV's anti-seizure effects appear to primarily result from interaction with SV2A (Kaminski et al., 2009).

The markedly attenuated LTP observed in iTAT animals (Fig. 4) (Fitting et al., 2013) might result from potentiation of NMDA receptors via a TAT-induced phosphorylation that occluded LTP (Haughey et al., 2001; Kim et al., 2008; King et al.,

2010). LEV-mediated inhibition of glutamate release may balance this potentiation. Alternatively, an increase in inhibitory tone to compensate for the increased sEPSC frequency observed in prefrontal cortex (Jacobs et al., 2019) and hippocampus (Fig. 1) could suppress LTP, although we did not detect an increase in sIPSC frequency in iTAT mice (Fig. 3). The dramatic reversal of the TAT-induced reduction of LTP by LEV is consistent with the improved cognitive performance observed in iTAT animals given LEV.

Previous studies have shown that TAT expression impaired performance in the Barnes (Carey et al., 2012) and Morris water mazes (Harricharan et al., 2015). We found that LEV significantly improved the performance of iTAT animals in the Barnes maze. LEV decreased latency to find the goal, reduced the number of errors, and improved cognitive scores based on search strategy (Figs. 7 and 8). In other disease models, LEV was the only AED that improved executive function and spatial memory (Belcastro et al., 2007; Sanchez et al., 2012), especially for tasks requiring the hippocampus (Bakker et al., 2015). However, we found that LEV did not prevent all TAT-induced deficits. Notably, LEV treatment did not prevent impairment in the probe test. This might suggest that LEV only rescues short-term memory, but iTAT + LEV mice performed the task better than iTAT + SAL mice on the first trial of each acquisition day. The discrepancy between the 4-day acquisition of the task that was enhanced by LEV versus the lack of LEV effect in the probe test may be due to stress induced by the probe test, as there is no way to escape. TAT induces an anxiogenic phenotype (Paris et al., 2014; Hahn et al., 2016) that is unlikely to be alleviated by LEV because LEV can be anxiogenic (Kanner and Bicchi, 2022). The combined results showing that LEV

improved several performance measures in the Barnes maze (latency, errors, and cognition) provide convincing evidence that LEV improves spatial memory in iTAT mice even though the deficit in the single trial probe test was not reversed. The observation that LEV rescues some aspects of impaired behavioral function but not others, despite dramatic restoration of sEPSC frequency and LTP, suggests that TAT disrupts function by multiple mechanisms, not all of which are mediated via increased excitatory synaptic transmission.

Based on the increased sEPSC frequency in iTAT mice and restoration of synaptic and cognitive function by the antiepileptic drug LEV, we explored the possibility that TAT expression would produce epileptiform spikes and possibly electrographic seizures. However, overt EEG abnormalities were not detected in mice expressing TAT (Fig. 5). Only when challenged with kainate was a hyperexcitable, increased seizure-propensity, profile observed in iTAT mice (Fig. 6). While it is possible that depth electrodes in the hippocampus failed to detect seizures in different brain regions, considering the changes in hippocampal synaptic function and spatial learning, a more reasonable explanation is that LEV prevents subtle alterations in glutamatergic synaptic transmission. However, in a clinical trial examining LEV effects on cognition in AD, only when patients were stratified by seizure status was LEV found to elicit positive effects on spatial memory and executive function (Vossel et al., 2021). Both overlapping and distinct neurophysiological aberrations were observed in AD and HAND patients using magnetoencephalography (Meehan et al., 2023). Perhaps the effects of LEV are graded with more dramatic effects observed in subjects with more enhanced network excitability, suggesting mice exposed to TAT for a prolonged period would develop overt

EEG abnormalities. Indeed, neurocognitive impairment is more pronounced in older PLWH (Jaqua et al., 2022) although comorbidities also contribute (Heaton et al., 2023).

LEV is approved by the US Food and Drug Administration and is better tolerated and more efficacious than other AEDs for many types of seizures (Contreras-García et al., 2022). It is currently used to treat overt epilepsy in HIV patients (Siddiqi and Birbeck, 2013). Additional preclinical studies would help determine if clinical studies using LEV to treat HAND are warranted. One hour treatment with LEV reversed the elevated sEPSC frequency in hippocampal slices from iTAT animals (Fig. 2) suggesting that LEV can reverse existing neuropathological changes. However, additional studies that assess the effects of LEV on cognitive function in older animals with established neuropathology would be beneficial. Additional EEG studies following prolonged exposure to HIV-1 TAT are also warranted. If EEG abnormalities develop in older animals, EEG may be a useful biomarker for identifying HAND patients that would respond well to treatment with LEV. There are a number of animal models for HAND in which LEV could be tested, including live virus-infected primate and humanized-mouse models (Mallard and Williams, 2018), the mouse tropic ecoHIV model (Potash et al., 2005), and rodent models that express individual (Kim et al., 2003; Thaney et al., 2018) and multiple HIV proteins (McLaurin et al., 2018; Keledjian et al., 2023). Synaptic loss observed in HAND (Everall et al., 1999) and animal models of HAND (Irollo et al., 2021) results from aberrant glutamatergic signaling due to elevated extrasynaptic glutamate (Vartak-Sharma et al., 2014), sensitization to synaptic glutamate (Bellizzi et al., 2005) or increased frequency of glutamatergic neurotransmission (Fig. 1) and thus, multiple mechanisms contributing to cognitive decline are potentially sensitive to LEV treatment.

Lastly, we studied effects of LEV on male iTAT mice because the effects of TAT are less robust in females (Hahn et al., 2015). Clearly, further testing in models with established HAND-like effects in females are warranted.

In conclusion, we have identified LEV as a potential therapeutic that attenuates aberrant glutamatergic synaptic activity and prevents cognitive impairments in an animal model of HAND; LEV attenuated excitatory signaling, improved synaptic plasticity, reduced seizure susceptibility, and preserved cognition in iTAT mice.

Chapter Three: Concluding Remarks

I: Summary of current study and context within literature

HAND is a debilitating neurological disease that currently has no treatment. The HIV protein TAT is thought to be responsible and has been studied in a transgenic mouse model that recapitulates cognitive deficits seen in HAND. Here, for the first time, we showed that an anti-epileptic drug, LEV, was able to prevent TAT-induced molecular and behavioral impairments: TAT increased sEPSCs, impaired LTP, reduced seizure threshold, and impaired learning acquisition in the Barnes maze; each of these was protected by LEV.

TAT was first shown to cause hippocampal apoptosis, dendritic loss, neurotoxicity, and neuroinflammation in a novel transgenic mouse model (Kim et al., 2003). Additional studies have reproduced and established these effects of TAT, as well as increased excitatory signaling (Jacobs et al., 2019), dampened synaptic plasticity (Fitting et al., 2013), and impaired performance in the Barnes (Carey et al., 2012) and Morris water mazes (Harricharan et al., 2015). Because of this evidence and its persistence despite cART, TAT is thought to be responsible for HAND symptomology.

Despite extensive research on the actions of TAT, there are no treatments to combat TAT's neurotoxic effects. This prompts investigation into common mechanisms of neurodegeneration, such as AD, which bears resemblance to neurodegeneration in HAND (Rempel and Pulliam, 2005; Moore et al., 2006; Ru and Tang, 2017; Li et al., 2022). In an AD-model mouse, LEV protected against neurological and behavioral deficits (Sanchez et al., 2012). Similarly, a clinical trial using LEV in AD patients showed success in attenuating cognitive decline in those with epileptiform activity (Vossel et al., 2021). Mild cognitive impairment, which is thought to be a steppingstone to AD, is

improved by LEV treatment (Bakker et al., 2012), consistent with our findings that mild impairments induced by TAT are also alleviated by LEV administration. Neurodegeneration is a hallmark of HAND (Everall et al., 1999; Moore et al., 2006; Ru and Tang, 2017), providing support for using LEV to protect from TAT-induced toxicity. Here, we found that LEV protected cognition in iTAT mice, suggesting a common underlying mechanism of neurodegeneration that is exacerbated by epileptic activity.

II: LEV mechanism of protection

Due to its broad mechanistic activity, there are a multitude of ways that LEV could preserve plasticity and cognition in this model. The simplest explanation is that LEV reverses or prevents TAT from causing SV2A dysfunction. This mechanism is consistent with attenuation of the TAT-induced increase in sEPSC frequency observed in this study (Fig. 1). An action on vesicle trafficking might also explain the slow (> 1 h) onset of action in *in vitro* experiments (Fig. 2), although the delayed effects could also result from a slow reversal of network changes induced by TAT. Attenuation of vesicle priming by LEV might create a use-dependent effect that would explain relative sparing of sEPSC frequency in recordings from WT animals (Fig. 1). However, if TAT disturbed SV2A function, TAT mice would likely have severe seizures, and, at the very least, abnormal EEGs, which were not detected in our study (Fig. 5). Only when challenged with kainate was a hyperexcitable, increased seizure-propensity profile observed in iTAT mice (Fig. 6).

The evidence of TAT disturbing glutamate cycling (Brailoiu et al., 2008; Saylor et al., 2016; Jacobs et al., 2019; Cirino and McLaughlin, 2021) and increasing

glutamatergic release (Fig.1) suggest a more subtle disruption of glutamatergic signaling that might take more time to develop into subclinical epileptic activity. For example, it is possible that LEV increases glutamatergic transporter expression (Ueda et al., 2007), which may aid in clearing the excess synaptic glutamate induced by TAT. This could explain the markedly attenuated LTP observed in iTAT animals (Fig. 4) (Fitting et al., 2013), which might result from potentiation of NMDA receptors via a TAT-induced phosphorylation that occludes LTP (Haughey et al., 2001; Kim et al., 2008; King et al., 2010). LEV-mediated inhibition of glutamate release may balance this potentiation.

The neuroprotection provided by LEV in this model could also suggest a more indirect interaction of TAT and LEV. LEV has many additional peripheral mechanisms that may aid in its anti-epileptic and neuroprotective properties. For instance, TAT disrupts calcium levels by increasing release from intracellular stores (Haughey et al., 1999), which could be attenuated by LEV (Angehagen et al., 2003). LEV is also anti-inflammatory (Itoh et al., 2019) and could combat the pro-inflammatory cytokines upregulated by TAT (Mediouni et al., 2015b), which are known to increase spontaneous excitatory signaling (Galic et al., 2012).

Additionally, heightened amyloid-beta levels, a hallmark of AD, are sufficient to cause seizures (Palop et al., 2007). Amyloid-beta has been found in hippocampal post-mortem tissue from PLWH and HIV-transgenic rats (Li et al., 2022). Further, TAT has been found to inhibit neprilysin, the enzyme that degrades amyloid-beta, allowing it to accumulate and cause epileptic activity and synapse loss in HIV (Rempel and Pulliam, 2005). Intriguingly, LEV decreases amyloid precursor protein levels, thereby limiting the

amount of amyloid-beta that can be created (Kasatkina et al., 2022), but this is the only point in the pathway that LEV acts upon; even long-term LEV administration does not alter amyloid-beta levels (Sanchez et al., 2012). Therefore, if amyloid-beta is already present in large amounts, LEV can only prevent further damage from occurring. This could explain why prophylactic LEV administration protected from molecular and behavioral deficits (Figs. 1, 2, 4, 7, and 8). It also cannot be ruled out that LEV acts initially on SV2A (as suggested by Fig. 2), while providing long-term neuroprotection through multiple synergistic mechanisms.

III: Use of LEV in HAND

LEV is typically prescribed as an anti-seizure medication because it rapidly attenuates excess glutamate release (Steinhoff and Staack, 2019), but it also has many other anti-inflammatory and neuroprotective mechanisms of action that have yet to be fully elucidated (Contreras-García et al., 2022). Recent evidence has shown its promise in cognitive protection in neurodegeneration. This is because, in contrast to other commonly used seizure medications, LEV is one of the only AEDs that improves executive functioning and spatial memory (Belcastro et al., 2007; Sanchez et al., 2012), especially when the hippocampus is required (Bakker et al., 2015).

LEV is an attractive AED to use for neuroprotection in HAND, as it is better tolerated and more effective than other AEDs (Contreras-García et al., 2022) and is already used to treat epilepsy in HIV patients (Siddiqi and Birbeck, 2013). Whether it improves cognitive outcomes in this population remains to be seen. As found in our study (Fig. 2) and others (Birnstiel et al., 1997; Sanchez et al., 2012), LEV preferentially

inhibits seizure activity and does not affect normal signaling, making it safe to use as a protective treatment. Our study suggests that treating with LEV as early as possible in disease progression is crucial for limiting damage and preserving cognition. Pending success in other animal models of HIV, perhaps PLWH could be screened for abnormal EEG activity, like that found recently in AD patients (Vossel et al., 2016), before HAND develops, and identified for LEV treatment eligibility.

IV: Use of LEV in other neurodegenerative diseases

The idea that AEDs or LEV can provide neuroprotection beyond dampening seizures is still emerging; there is limited research on LEV's ability to afford protection in neurodegenerative disease states, including HAND. LEV was found to improve cognition in AD patients with epileptic activity; there was no effect on AD patients with no epileptic activity (Vossel et al., 2021), suggesting that LEV can only elicit its beneficial effects on cognition by blocking seizures. An ongoing clinical trial testing whether LEV can rescue mild neurocognitive impairment in PD provides precedence that LEV may alleviate cognitive deficits (Dissanayaka et al., 2023). Like the animal models of AD, LEV is the only AED considered helpful for cognition in PD; carbamazepine and valproate downregulate dopaminergic neurons and inhibit dopamine receptor function (Basselin et al., 2008; Ramadan et al., 2011), which would exacerbate a state of dopaminergic degeneration in PD. In both Huntington's disease (HD) and multiple sclerosis, LEV alleviated involuntary movements (de Tommaso et al., 2005) and tremors (Hawker et al., 2003; Solaro et al., 2020), while cognition was either unchanged or untested. Overall, the idea of AEDs, especially LEV, improving cognition

is still very new, but clinical trials using LEV in different neurodegenerative disease states will soon provide insight.

V: Caveats and limitations

Although the results of this study demonstrating LEV's ability to preserve cognition in HAND are encouraging, there are a few caveats and limitations. First, to our surprise, we did not detect overt seizure activity or interictal spiking on EEGs, or any significant changes to power spectral density (Fig. 5). This does not rule out that subclinical activity is occurring; we may not have been able to detect subtle changes in glutamatergic signaling, or they may develop more clearly with more chronic DOX administration. Additionally, we did not see alterations to spontaneous inhibitory signaling (Fig. 3). Other studies have shown that TAT quickly induces an initial spike in IPSC frequency of cultured neurons (Brailoiu et al., 2008). Our study employed *ex vivo* brain slices, which provide an architecture and environment similar to the brain *in vivo*. The electrophysiology experiments in our study were also conducted following 7 days of DOX administration, in which changes to synaptic scaling could have occurred and may cloud the interpretation of changes to frequency. Overall, the increase in sEPSCs without a compensatory increase in sIPSCS supports an excitatory/inhibitory imbalance.

Interestingly, all metrics in the Barnes maze were rescued by LEV except the probe test. This would initially suggest that LEV only rescues short-term memory, but iTAT + LEV mice performed the task better than iTAT + SAL mice on the first trial of each acquisition day (data not shown). These conflicting results may be due to the probe test inducing stress, as there is no way to escape. Others have found that TAT

induces an anxiogenic phenotype (Paris et al., 2014; Hahn et al., 2016) that is unlikely to be alleviated by LEV, as LEV can also be anxiogenic (Kanner and Bicchi, 2022). Although surprising, the combined results of the latency, errors, and cognition, along with the rescue of glutamatergic signaling and plasticity, are more compelling than the single trial in the probe test.

While the iTAT transgenic mouse model reproduces symptoms of HAND, it does not generate the full spectrum of HIV pathology. Alternatively, it is imperative to know what TAT does alone. TAT remains in the CNS despite cART, so understanding how it causes damage will best inform therapeutic approaches. Similarly, TAT is in the CNS of PLWH much longer than one week, so we can only make conclusions about early stages of HAND.

LEV was given prophylactically one week before beginning DOX injections, due to the need for mice to recover from the osmotic minipump surgery for 5-7 days. PLWH could not start LEV prophylactically, so LEV needs to reverse existing deficits in more chronic models of HAND before moving to clinical trials. We show limited data concerning reversal of sEPSC frequency *in vitro* (Fig. 2) that suggests LEV produces the same therapeutic effects within an hour, at least at the molecular level. Lastly, we studied effects of TAT on male mice, as TAT phenotypes may be less robust in females (Hahn et al., 2015).

VI: Future directions

This exciting work gives rise to many possible avenues for further research. Further work needs to be conducted in pre-clinical trials before moving to the clinical

setting. LEV's ability to afford protection should be tested in more chronic models of TAT, where mice are administered DOX through chow for several months (Jacobs et al., 2019), as well as other models of HAND and HIV, such as the mouse-tropic EcoHIV model (Potash et al., 2005), the murine HIV model of TAT (Reid et al., 2001) and those expressing multiple HIV proteins (Keledjian et al., 2023). Since PLWH would not be able to take LEV prophylactically, it also needs to be demonstrated that LEV's therapeutic action is not limited to a prophylactic model and should be tested after several months of TAT and/or HIV expression.

Additional EEG studies in more chronic models are also warranted since these experiments were performed at an early-stage expression of TAT and abnormalities may not be evident without more chronic exposure to TAT. It is also possible that more intensive EEG studies involving additional electrodes or multiple brain areas could reveal subclinical activity and provide some insight on mid-stage disease state. This would be especially important to determine, as EEG abnormalities could be a powerful biomarker for HAND and would identify patients that should begin LEV administration to maintain cognition.

LEV's therapeutic mechanism could be further elucidated by studying heterozygous SV2A knock out mice, as in (Kaminski et al., 2009), crossed with iTAT mice and testing whether cognition is still preserved after LEV administration. Additionally, therapies that target one of LEV's multiple mechanisms, such as anti-inflammatory action, could be used alongside LEV to see whether they also improve cognition in iTAT mice. Lastly, further investigation into HAND phenotypes and treatment with LEV in females is warranted.

In conclusion, we have demonstrated LEV as the first potential therapeutic to address hyperexcitability and cognitive impairments in HAND: LEV attenuates excitatory signaling, improves synaptic plasticity, protects from seizure susceptibility, and preserves cognition in iTAT mice. This study provides support for testing LEV's neuroprotection in other models of HIV. LEV deserves more study in neurodegenerative disease, as there may be a common underlying mechanism of excitotoxicity or epileptic activity that exacerbates cognitive impairment. If so, it is crucial to identify PLWH that may have abnormal EEGs so that LEV can be administered as early as possible to slow cognitive decline.

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