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RESEARCH ARTICLE

Amisulpride as a potential disease-modifying drug in the treatment of tauopathies

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Abstract

INTRODUCTION: Hyperphosphorylation and aggregation of the microtubule-associated protein tau cause the development of tauopathies, such as Alzheimer's disease and frontotemporal dementia (FTD). We recently uncovered a causal link between constitutive serotonin receptor 7 (5-HT7R) activity and pathological tau aggregation. Here, we evaluated 5-HT7R inverse agonists as novel drugs in the treatment of tauopathies.

METHODS: Based on structural homology, we screened multiple approved drugs for their inverse agonism toward 5-HT7R. Therapeutic potential was validated using biochemical, pharmacological, microscopic, and behavioral approaches in different cellular models including tau aggregation cell line HEK293 tau bimolecular fluorescence complementation, primary mouse neurons, and human induced pluripotent stem cell–derived neurons carrying an FTD-associated tau mutation as well as in two mouse models of tauopathy.

RESULTS: Antipsychotic drug amisulpride is a potent 5-HT7R inverse agonist. Amisulpride ameliorated tau hyperphosphorylation and aggregation in vitro. It further reduced tau pathology and abrogated memory impairment in mice.

DISCUSSION: Amisulpride may be a disease-modifying drug for tauopathies.

KEYWORDS
amisulpride, antipsychotics, dementia, inverse agonists, serotonin receptor 5-HT7R, tau, tauopathies

1 | BACKGROUND

A common feature of multiple neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease, and frontotemporal dementia (FTD), is the formation and deposition of protein aggregates inside and outside of neurons, accompanied by progressive neuronal loss and cognitive decline. In particular, aggregation of microtubule-associated protein tau is causative for the development and progression of so-called tauopathies, such as AD, FTD, corticobasal degeneration, and progressive supranuclear palsy.[1]

In neurons, tau is bound to microtubules to regulate microtubule network stability and dynamics, as well as axonal transport, extension, and maturation.[2] The affinity of tau for binding microtubules is tightly regulated and can be influenced by mutations in the *MAPT* gene or post-translational modifications of tau.[3] In particular, hyperphosphorylation of tau results in detachment from and destabilization of the microtubule network.[1] This also affects axonal transport, proteasomal degradation pathways, and mitochondrial and synaptic functions.[4–6] Pathological hyperphosphorylation facilitates tau aggregation and its accumulation into filamentous structures, so-called neurofibrillary tangles (NFTs), which correlate with neurotoxicity.[7,8] Despite the efforts that have been made in understanding tau-mediated pathomechanisms, no effective therapy is available that can stop or reverse the progressive course of tauopathies.

We recently uncovered a causal link between serotonin receptor 7 (5-HT7R) and tau hyperphosphorylation, tau aggregation, neuronal death, and cognitive impairment.[9] 5-HT7R is highly expressed in the cerebral cortex and hippocampus, areas primarily affected by AD and FTD,[10] and plays an important role in higher cognitive functions, including memory formation and learning.[11,12] 5-HT7R–mediated signaling is activated upon binding of the neurotransmitter serotonin. Moreover, 5-HT7R possesses a high constitutive activity enabling the initiation of various signaling pathways even in the absence of serotonin.[13–15] We found that constitutive activity of 5-HT7R leads to activation of the tau kinase cyclin-dependent kinase 5 (CDK5) via a direct, G protein-independent mechanism. Importantly, blockade of 5-HT7R constitutive activity by the inverse agonist SB-269970 abrogated the deleterious effects on tau hyperphosphorylation and aggregation, emphasizing its therapeutic potential.[9]

As SB-269970 is a research compound that is not permitted for use in humans, we screened numerous approved drugs for inverse agonism toward 5-HT7R. We identified several antipsychotics, including clozapine, amisulpride, and lurasidone, that possess a high binding affinity for 5-HT7R and act as its inverse agonists. The therapeutic potential of amisulpride was confirmed in vitro and in vivo using several models, including HEK293 tau bimolecular fluorescence complementation (tau-BiFC) cells, human from induced pluripotent stem cell (iPSC)–derived cortical neurons and two different mouse models of tauopathy, including overexpression of the human tau[R406W] mutant or knock-in of the human tau[P301L] mutant.

2 | METHODS

For detailed protocols see supporting information.

2.1 | Drugs

The following drugs were used: SB-269970 hydrochloride (Tocris), amisulpride (Selleckchem), mianserin (Selleckchem), clozapine (Selleckchem), lurasidone (Selleckchem), tiapride (TCI), or vortioxetine (Selleckchem).

2.2 | Cells

2.2.1 | HEK293 tau-BiFC cells

The HEK293 cell line that stably expresses the tau-BiFC sensor was cultured as previously reported.[16,17]

2.2.2 | Primary cortical neurons

Primary cortical neurons were extracted from the prefrontal cortex of newborn (postnatal day P0–P2) wild-type (WT) mice of both sexes from strain C57BL/6JHanZtm using an optimized protocol previously published.[9] On day 10, cells were infected with 10^8 copies of Adeno-associated virus (AAV)–enhanced green fluorescent protein (eGFP)–tau[R406W] per 12-well and treated for 3 days.

2.2.3 | Human cortical neurons from iPSC-derived neural stem cells

Human cortical neurons carrying the tau[R406W] mutation as well as isogenic controls were differentiated from iPSC-derived neural stem cells (NSCs) as previously reported.[18] At day 35 of differentiation, neurons were treated for 3 days.

RESEARCH IN CONTEXT

Systematic Review: Pathological tau aggregation is a hallmark of multiple neurodegenerative diseases collectively termed tauopathies. Tau hyperphosphorylation is a significant factor driving tau aggregation leading to impaired axonal transport, synaptic dysfunction, and neuronal death. Recent studies have demonstrated that the constitutive activity of serotonin receptor 7 (5-HT7R) boosts tau-mediated pathology.

Interpretation: Our findings demonstrate that pharmacological targeting of 5-HT7R by inverse agonists represents a new strategy for the treatment of tauopathies. In particular, treatment with the antipsychotic drug amisulpride ameliorates various aspects of tau pathology, including abrogation of hyperphosphorylation, tangle formation, apoptosis, and memory deficits, making it a strong candidate for drug repurposing.

Future Directions: The next step will include initiating a phase II trial to study the therapeutic effects of amisulpride for treatment of tauopathies in humans. Medicinal chemistry refinement studies will contribute to the development of more potent and highly selective 5-HT7R inverse agonists based on amisulpride.

2.3 | Animals

2.3.1 | C57BL/6J WT mice

All mice were cared for and treated strictly following the ethical animal research standards defined by the Directive 2010/63/EU and were approved by the Ethical Committee on Animal Health and Care of Saxony-Anhalt state, Germany (license numbers: 42502-2-1343 DZNE and 42502-2-1346 DZNE) or the Lower Saxony's "Landesamt für Verbraucherschutz und Lebensmittelsicherheit."

2.3.2 | Tau^{P301L}-BiFC model

Tau^{P301L}-BiFC transgenic mice were bred and maintained as previously described.[19] Animal protocols were approved and followed the principles and practices outlined by guidelines of the Institutional Care and Use Committee of the Korea Institute of Science and Technology.

3 | RESULTS

3.1 | Antipsychotic drug amisulpride is an inverse agonist of 5-HT7R

According to structural similarity to the highly specific 5-HT7R inverse agonist SB-269970, we selected the antipsychotics amisulpride, clozapine, and lurasidone and the antidepressants mianserin and vortioxetine for pharmacological analysis. In particular, we analyzed their potential to inhibit constitutive 5-HT7R activity, which not only modulates CDK5 functions but also activates adenylyl cyclase, which results in an increase in cyclic adenosine monophosphate (cAMP) production. These experiments revealed that SB-269970, amisulpride, mianserin, clozapine, and lurasidone act as potent 5-HT7R inverse agonists (Figure 1A, Table S1 in supporting information). In contrast, vortioxetine is a 5-HT7R antagonist that did not block constitutive activity but effectively inhibited the agonist-induced activity of 5-HT7R (Figure 1A; Figure S1A in supporting information). Tiapride, a selective dopamine D2 and D3 receptor antagonist with no affinity toward 5-HT7R,[20] also failed to block constitutive 5-HT7R activity (Figure S1B). The half-maximal effective concentration for inhibition of basal cAMP production (IC_{50}) was lowest for SB-269970 (11 ± 3 nM), followed by amisulpride (74 ± 20 nM), clozapine (226 ± 22 nM), mianserin (330 ± 1 nM), and lurasidone (445 ± 241 nM). This indicates that amisulpride, which has a similar chemical structure as SB-269970 (Figure 1B), is the most potent 5-HT7R inverse agonist. Importantly, amisulpride had no effect on basal cAMP levels in cells without 5-HT7R (Figure S1C) and did not inhibit constitutive activity of 5-HT4R (Figure 1C–F), indicating that amisulpride is a specific inverse agonist of 5-HT7R.

3.1.1 | 5-HT7R inverse agonists decrease 5-HT7R–induced tau aggregation in HEK293 tau-BiFC cells

To study the potential of the selected drugs in preventing tau aggregation, we used the HEK293 tau-BiFC cell model.[16] This cell line stably expresses human WT tau protein fused to either the non-fluorescent amino (N)- or carboxy (C)-terminal part of the Venus protein. Monomeric tau does not exhibit any fluorescence, but Venus fluorescence becomes visible upon tau hyperphosphorylation and subsequent aggregation (Figure S2A in supporting information). HEK293 tau-BiFC cells were transfected with a plasmid cloning DNA vector encoding HA-tagged 5-HT7R and treated with 50 μM amisulpride, mianserin, clozapine, or lurasidone for 24 hours (Figure 2A). Vortioxetine and tiapride were used as negative controls. Under basal conditions, 5-HT7R expression induced a more than 5-fold increase in BiFC fluorescence, reflecting strong receptor-mediated tau aggregation (Figure 2B,C). Blockade of constitutive receptor activity by SB-269970 significantly reduced tau aggregation. Importantly, treatment with amisulpride, lurasidone, mianserin, or clozapine decreased Venus fluorescence in 5-HT7R–expressing cells to the same extent as SB-269970. The 5-HT7R antagonist vortioxetine seemed to have cytotoxic effects at this concentration, as vortioxetine treatment significantly decreased cell numbers leading to a reduction of BiFC fluorescence below basal levels (Figure 2C and Figure S2B). In contrast, tiapride did not prevent 5-HT7R–induced tau aggregation, indicating that the observed effects are 5-HT7R-specific.

To define the effective concentrations for preventing tau aggregation and to study the impact of selected drugs on cell viability and

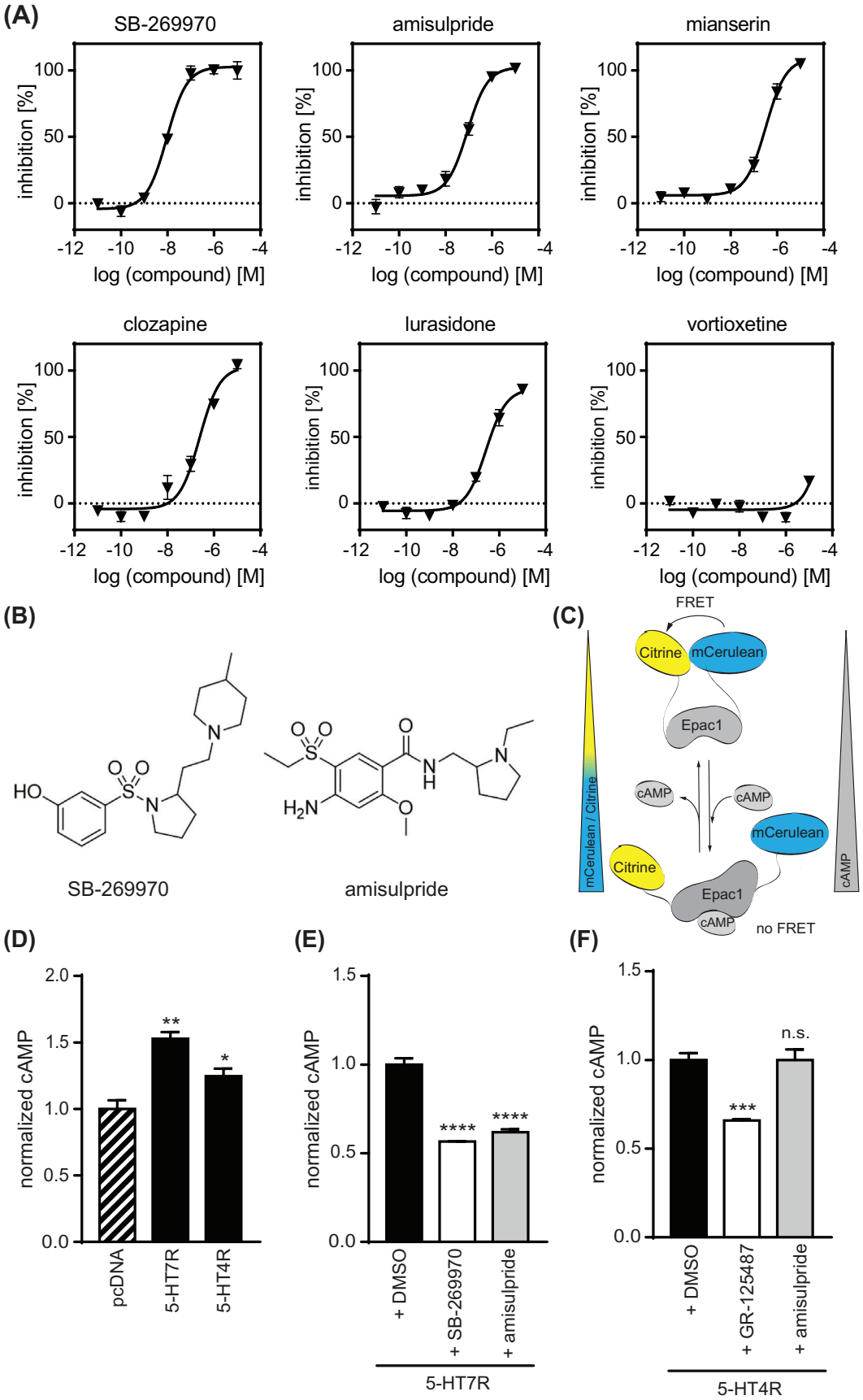

FIGURE 1 Amisulpride is an inverse agonist for 5-HT7R. A, Dose–response curves for inhibition of constitutive 5-HT7R activity by SB-269970, amisulpride, mianserin, clozapine, lurasidone, and vortioxetine. HEK293 cells expressing 5-HT7R were treated with the indicated compounds in triplicats at concentrations varying from 10^{-11} to 10^{-5} M. Afterward, basal cAMP levels were measured. B, Structural formula of SB-269970 or amisulpride. Both contain ethylsulfonyl- and benzoic acid moieties. C, Scheme of the FRET-based cAMP biosensor. High cAMP

proliferation, we analyzed the dose–response relationship of these drugs for BiFC fluorescence and cell viability. SB-269970 was most effective in preventing tau aggregation ($EC_{50} = 0.2 \pm 0.1$ μM), followed by mianserin ($EC_{50} = 0.8 \pm 0.2$ μM), lurasidone ($EC_{50} = 0.6 \pm 0.1$ μM), amisulpride ($EC_{50} = 1.9 \pm 0.2$ μM), and clozapine ($EC_{50} = 2.3 \pm 0.2$ μM; Figure 2C and Figure 2E, Table S2 in supporting information). Notably, after treatment with SB-269970 or amisulpride, cell proliferation was similar to that of control cells, whereas treatment with all other drugs significantly reduced cell numbers. Mianserin, clozapine, lurasidone, and vortioxetine influenced cell proliferation at low concentrations, whereas SB-269970, amisulpride, and tiapride had no inhibitory effects on the cell number, even at concentrations of 100 μM (Figure S2B,C and Table S2). To exclude cytotoxic effects, vortioxetine was used at a concentration of 1 μM in all further experiments.

Biochemical analysis revealed that expression of 5-HT7R dramatically increased the tau protein levels in HEK293 tau-BiFC cells (Figure 2D). Treatment of these cells with SB-269970, amisulpride, mianserin, or clozapine resulted in a significant decrease in tau levels without affecting 5-HT7R expression (Figure 2D and Figure S2D). Lurasidone-treated cells also showed reduced levels of total tau protein, but this effect was accompanied by decreased expression of 5-HT7R. In contrast, vortioxetine and tiapride had no effects on 5-HT7R–induced increase in tau. Of note, expression of *MAPT* mRNA was not significantly changed by any of the treatments (Figure S2E).

Although all tested 5-HT7R inverse agonists have been shown to block tau aggregation, we focused on amisulpride for the following reasons: (1) amisulpride possesses the highest inverse agonistic potential toward 5-HT7R, (2) 5-HT7R expression levels are not affected upon amisulpride treatment, and (3) it can be applied in concentrations up to 100 μM without any cytotoxic effect.

3.2 | Amisulpride reduces the formation of insoluble tau aggregates in HEK293 tau-BiFC cells

Next, we investigated the impact of the selected drugs on the formation of tau-BiFC aggregates. Though HEK293 tau-BiFC cells expressing mCerulean (negative control) showed almost no tau-Venus fluorescence, 5-HT7R–expressing cells exhibited pronounced accumulation of fluorescent tau aggregates, similar to those obtained in cells treated with forskolin, which was used as positive control[21] (Figure 3A). Treatment of cells expressing 5-HT7R with SB-269970 or amisulpride effectively prevented the formation of tau aggregates, whereas vortioxetine and tiapride had no effect (Figure 3A).

As shown in Figure 3B, 5-HT7R expression increased tau phosphorylation, and treatment with SB-269970 or amisulpride led to a significant reduction in phospho-tau levels (Figure 3B). In contrast, treatment with vortioxetine or tiapride did not influence 5-HT7R–induced tau phosphorylation. To distinguish between soluble monomeric and oligomeric tau, as well as insoluble tau aggregates, we applied the sarkosyl fractionation approach. In line with our previous study, cells expressing 5-HT7R had elevated levels of tau particularly in the sarkosyl-soluble and insoluble fraction, indicating the aggregation of tau in higher order oligomers and fibrillary tangles (Figure 3C).[9] Treatment of 5-HT7R–expressing cells with SB-269970 or amisulpride reduced tau levels, particularly in the insoluble fraction, whereas the amount of soluble tau was unaffected. In contrast, in cells treated with vortioxetine or tiapride, tau levels remained unchanged in all fractions (Figure 3C). These data further support that amisulpride may prevent pathological tau hyperphosphorylation and aggregation.

3.3 | Amisulpride reduces tau pathology in cultured mouse neurons and in human iPSC-derived neurons expressing tau[R406W] mutant

Next, we validated the therapeutic effects of amisulpride on tau pathology in an in vitro model of tauopathy that comprises overexpression of the human tau[R406W] mutant in murine primary cortical neurons. This mutant is causally associated with FTD and Parkinsonism linked to chromosome 17 (FTDP-17), and it preferentially results in NFTs in cortical neurons.[22,23] Primary neuronal cultures derived from the prefrontal cortex (PFC) of mice were infected with an AAV vector encoding eGFP-tau[R406W] and treated with H_2O, SB-269970, amisulpride, vortioxetine, or tiapride for 3 days, followed by confocal live-cell imaging and quantitative analysis (Figure 4A and Figure S3A in supporting information). These experiments revealed that 18.6% ± 1.0% of the eGFP-tau[R406W]–expressing neurons were tangle-positive, and SB-269970 treatment reduced the formation of tau tangles (Figure 4B,C). More importantly, treatment of infected neurons with amisulpride significantly reduced the number of tangle-positive neurons to 8.0% ± 0.5%, whereas vortioxetine and tiapride did not prevent the formation of tau tangles (Figure 4C). Notably, the total number of cortical neurons and the cellular composition were not altered upon treatment (Figure S3B). Furthermore, the eGFP-tau[R406W] expression did not differ between different conditions (Figure S3C).

levels correlate with low FRET signal between mCerulean (FRET donor) and Citrine (FRET acceptor) calculated by donor-to-acceptor ratio. D, Basal cAMP levels measured in HEK293 cells transfected with pcDNA, (HA)5-HT7R, and (HA)5-HT4R. Signals are normalized to pcDNA-transfected cells. Data are presented as means + SEM ($N = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$, one-way ANOVA, Dunnett's multiple comparisons test. E, Normalized cAMP level in cells expressing 5-HT7R after treatment with vehicle (DMSO), 5 μM of the 5-HT7R-specific inverse agonist SB-269970 or amisulpride. F, Normalized cAMP level in cells expressing 5-HT4R after treatment with vehicle (DMSO), 5 μM of the 5-HT4R-specific inverse agonist GR-125487 or amisulpride. Signals are normalized to DMSO-treated cells. Data are presented as means + SEM ($N = 4$). $^{***}P < 0.001$; $^{****}P < 0.0001$, one-way ANOVA, Dunnett's multiple comparisons test. 5-HT7R, serotonin receptor 7; ANOVA, analysis of variance; cAMP, cyclic adenosine monophosphoate; DMSO, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; pcDNA, plasmid cloning DNA; SEM, standard error of the mean.

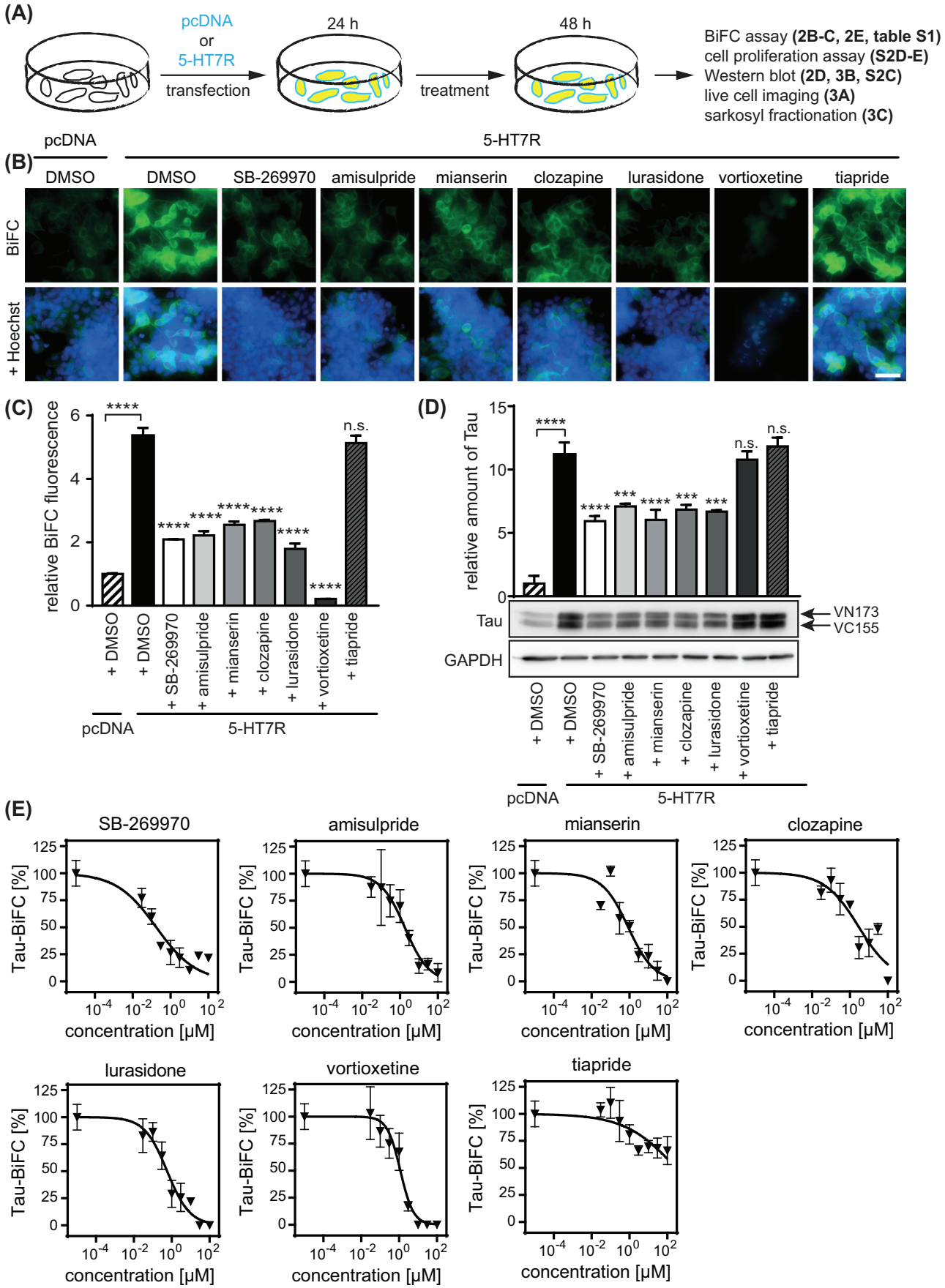

FIGURE 2 Amisulpride decreases 5-HT7R-induced tau aggregation in HEK293 tau-BiFC cells. A, HEK293 tau-BiFC cells were transfected with either pcDNA or 5-HT7R and treated with the indicated drugs after 24 hours for further 24 hours followed by analysis. B, Representative images showing pcDNA or (HA)5-HT7R transfected cells after treatment as indicated. BiFC fluorescence is shown in green, cell nuclei in blue.

The effect of amisulpride on tau aggregation in neurons was further analyzed using sarkosyl fractionation experiments. In infected untreated neurons, eGFP-tau[R406W] accumulated in the sarkosyl-soluble and insoluble fractions, indicating the formation of higher-order tau oligomers and tau aggregates (Figure 4D). Upon treatment with SB-269970, eGFP-tau[R406W] levels were reduced in both the sarkosyl-soluble and insoluble fractions, whereas the amount of tau in the soluble fraction was increased. This effect was even more pronounced in neurons treated with amisulpride, with tau accumulating mainly in the soluble fraction, whereas the sarkosyl-soluble and insoluble fractions contained almost no tau (Figure 4D). In contrast, neurons treated with vortioxetine or tiapride had a similar amount of eGFP-tau[R406W] in the sarkosyl-soluble and insoluble fractions compared to the untreated neurons (Figure 4D).

As tau oligomerization and aggregation are known to correlate with increased neuronal death,[7] we investigated whether amisulpride has a neuroprotective effect. In eGFP-tau[R406W]-infected, H_2O-treated neurons, 12.7% ± 0.5% of cells were apoptotic (Figure 4E; Figure S3D). Treatment with SB-269970 significantly decreased the number of apoptotic cells to 5.3% ± 0.8%. Similarly, treatment with amisulpride reduced the number of apoptotic neurons to 6.4% ± 0.7%, whereas vortioxetine and tiapride had no beneficial effects on tau[R406W]-induced neuronal apoptosis (Figure 4E). These data demonstrate that amisulpride treatment prevents aggregation of tau[R406W] mutant in PFC neurons and reduces neuronal death.

Having demonstrated favorable effects of amisulpride in murine cortical neurons, we investigated whether amisulpride ameliorates tau pathology in human neurons. To this end, we differentiated human iPSC-derived NSCs carrying the tau[R406W] mutation along with isogenic controls into cortical neurons, treated them with either vehicle (dimethyl sulfoxide), 50 µM amisulpride, or 50 µM tiapride for 3 days and analyzed tau phosphorylation levels (Figure 4F). Cultures derived from both NSC lines comprise mainly mature neurons as assessed by expression of neuron-specific proteins such as NeuN, MAP2, and synaptic markers including synapsin, synaptophysin, and postsynaptic density protein 95, as well as 5-HT7R (Figure S4A,B). Treatment of human cortical tau[R406W] neurons with amisulpride significantly decreased tau hyperphosphorylation at the multiple pathological phosphorylation sites (i.e., T181 and S202/T205) compared to vehicle-treated cells, while tiapride did not affect the phosphorylation levels (Figure 4G and Figure S4C,E in supporting information). Notably, we detected no significant effects of amisulpride on tau phosphorylation in isogenic controls (Figure S4C,D).

3.4 | Treatment with amisulpride decreases tau aggregation in vivo

To evaluate the effects of amisulpride on tau aggregation in vivo, we installed a cranial window during AAV-eGFP-tau[R406W] injection in the PFC, allowed the mice to recover and express tau[R406W] for 3 weeks, and monitored the eGFP-tau levels in vivo in mice treated with vehicle or amisulpride (daily intraperitoneal injections of 1 mg/kg) using two-photon microscopy (Figure S5A in supporting information). This approach allows for real-time analysis of tau aggregation in cortical neurons over the duration of drug administration. To analyze the neuronal integrity, we also visualized the brain's extracellular matrix by co-injecting an AAV-encoding hyaluronan and proteoglycan link protein 1 fused to fluorescent protein Scarlet (AAV-HAPLN1-Scarlet). In vehicle-treated mice, expression of eGFP-tau[R406W] continuously increased over time, indicating pronounced tau[R406W] aggregation in infected neurons (Figure S5B). In amisulpride-treated animals, tau accumulation was also visible within the first few days. However, after 16 days of amisulpride treatment, cells exhibited drastically reduced eGFP-tau[R406W] fluorescence, though the surrounding extracellular matrix remained intact, demonstrating neuronal integrity (Figure S5C). Notably, no changes were observed between the two groups in the structure of the extracellular matrix or the neuronal distribution within the PFC.

3.5 | Amisulpride ameliorates tau[R406W]-induced memory impairments in vivo

Using a similar treatment protocol (Figure 5A), we studied whether the beneficial effects of amisulpride on tau aggregation also improve tau-induced memory deficits in vivo. In an open field test, AAV-eGFP-tau[R406W]–infected mice did not exhibit significant differences from control animals in the distance traveled, whereas amisulpride-treated animals exhibited slightly reduced locomotor activity (Figure 5B). Time

Scale bar: 25 µm. C, Quantification of tau-BiFC fluorescence intensity in pcDNA or (HA)5-HT7R-transfected HEK293 tau-BiFC cells upon treatment with DMSO or 50 µM SB-269970, amisulpride, clozapine, lurasidone, mianserin, vortioxetine, or tiapride for 24 hours. Signals are normalized to DMSO-treated pcDNA-transfected cells. Data are presented as means + SEM ($N = 3$). $^{****}P < 0.0001$, n.s., not statistically different compared to 5-HT7R + DMSO, one-way ANOVA, Dunnett's multiple comparisons test. D, Representative western blot and quantification of HEK293 tau-BiFC cells transfected with pcDNA or (HA)5-HT7R after treatment with 50 µM SB-269970, amisulpride, clozapine, lurasidone, mianserin, or tiapride and 1 µM vortioxetine. Membranes were probed with total tau or GAPDH antibody. Signals are normalized to DMSO-treated pcDNA-transfected cells. Data are presented as mean + SEM ($N = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n.s. not statistically different compared to 5-HT7R + DMSO, one-way ANOVA, Dunnett's multiple comparisons test. E, Dose–response curves for inhibition of tau aggregation by SB-269970, amisulpride, clozapine, mianserin, lurasidone, vortioxetine, and tiapride. Tau-BiFC responses in cells transfected with (HA)5-HT7R and treated with the indicated drug at various concentrations were analyzed after 24 hours of treatment. A Prism's non-linear regression analysis was used to measure the EC_{50} value. 5-HT7R, serotonin receptor 7; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; pcDNA, plasmid cloning DNA; SEM, standard error of the mean; tau-BiFC, tau bimolecular fluorescence complementation.

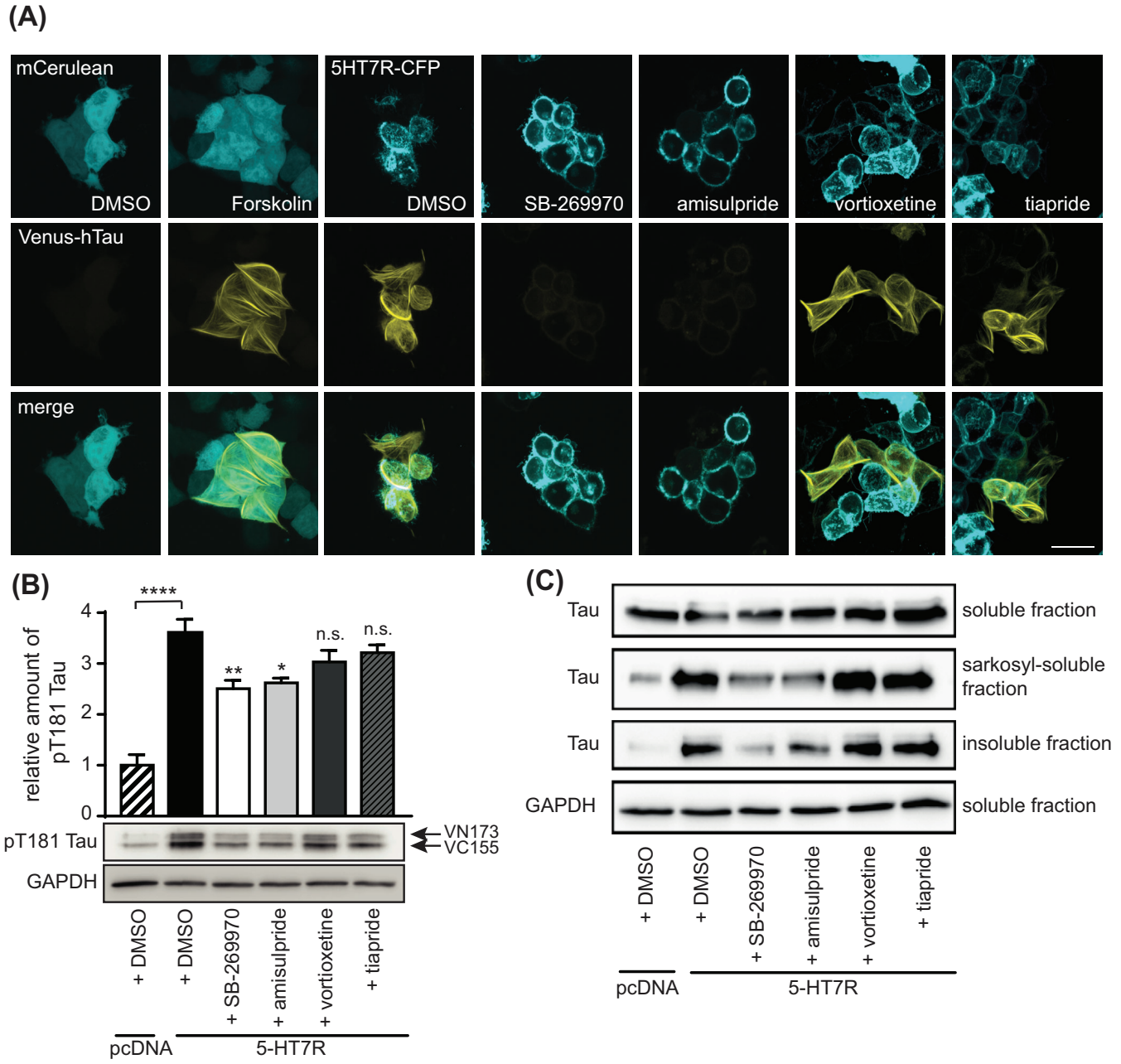

FIGURE 3 Amisulpride reduces hyperphosphorylation and formation of insoluble tau aggregates in HEK293 tau-BiFC cells. A, Representative confocal images of HEK293 tau-BiFC cells transfected with control vector (mCerulean, blue) or 5-HT7R (CFP-5-HT7R, blue) after treatment with 20 μM Forskolin, 50 μM SB-269970, or 50 μM amisulpride. Tau aggregates (Vn-Tau, yellow) presented in 5-HT7R-transfected or Forskolin-treated cells disappeared after treatment with SB-269970 and amisulpride. Scale bar: 20 μm. B, Representative western blot and quantification of HEK293 tau-BiFC cells transfected either with pcDNA or (HA)5-HT7R upon treatment as indicated. Membranes were probed with pT181 tau or GAPDH antibody. Signals are normalized to DMSO-treated pcDNA-transfected cells. Data are presented as means + SEM ($N = 4$). $^{*}P < 0.01$; $^{**}P < 0.01$; $^{****}P < 0.0001$ compared to 5-HT7R + DMSO, one-way ANOVA, Dunnett's multiple comparisons test. C, Representative western blot analysis with lysates from HEK293 tau-BiFC cells transfected with pcDNA or (HA)5-HT7R and treated with the indicated drugs after sarcosyl fractionation. Tau signals in soluble, sarkosyl-soluble, and sarkosyl-insoluble fractions were detected by a total tau antibody (5A6). GAPDH in the soluble fraction was used as a loading control. 5-HT7R, serotonin receptor 7; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; pcDNA, plasmid cloning DNA; SEM, standard error of the mean; tau-BiFC, tau bimolecular fluorescence complementation.

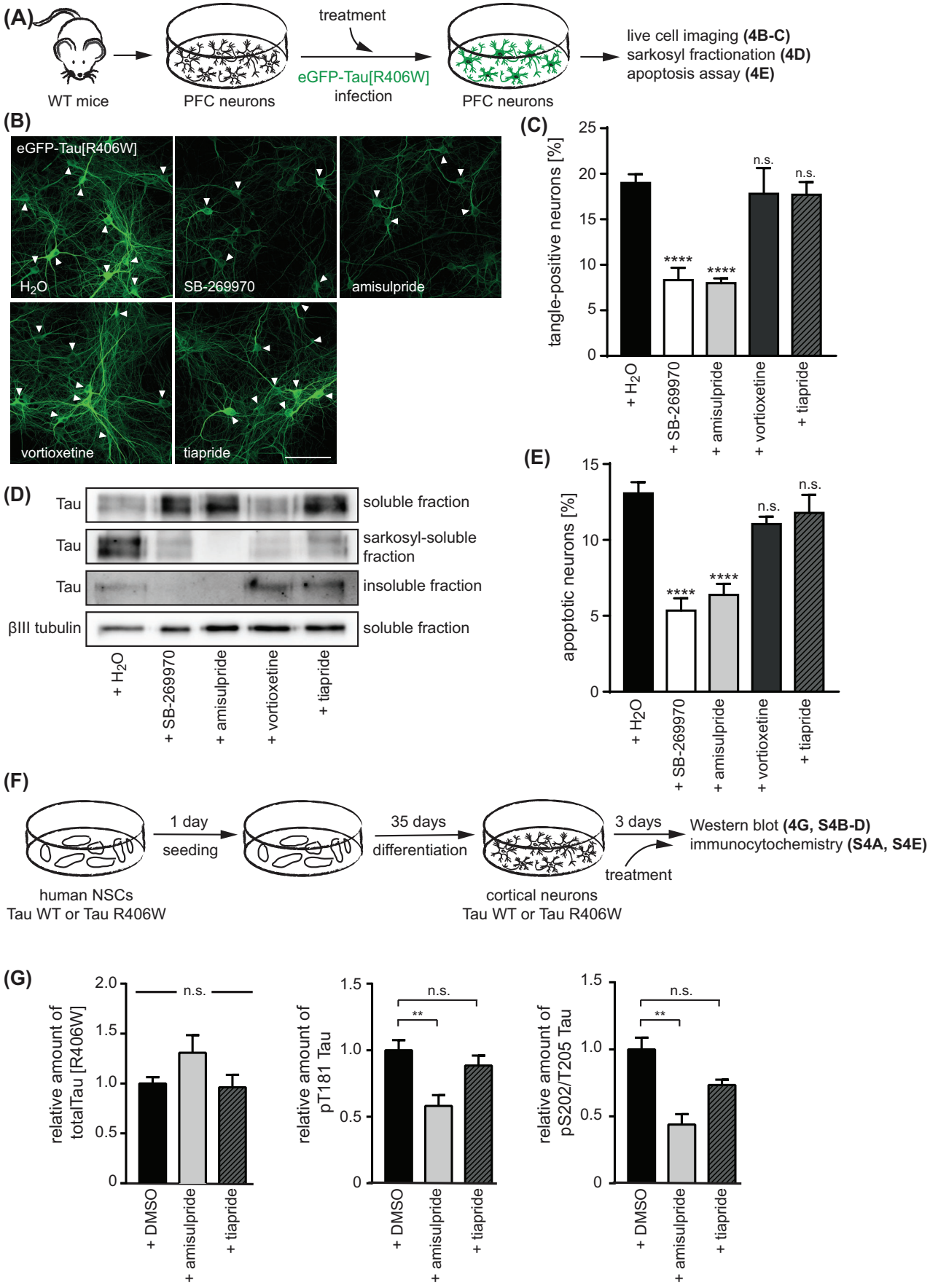

FIGURE 4 Amisulpride ameliorates tau pathology in mouse and human iPSC-derived cortical neurons expressing FTDP17-associated tau[R406W] mutant. A, Experimental design in mouse cortical neurons. Primary cortical neurons were infected with AAV-eGFP-tau[R406W] at DIV 10 and treated with H_2O, 100 nM SB-269970, 50 μM amisulpride, 1 μM vortioxetine, or 50 μM tiapride for 3 days and analyzed at DIV 13. B,

spent in the center and periphery was unchanged between all groups, indicating no influence of amisulpride on the level of anxiety. Finally, we applied the temporal order recognition memory test[24] to evaluate the cognitive functions in these mice (Figure 5C). Under control conditions, animals were able to discriminate between the two objects and spent more time with the less recently seen object (Figure 5D). Upon AAV-eGFP-tau[R406W] injection, mice exhibited impaired object recognition compared to mice injected with control AAV-eGFP (discrimination index 0.3% ± 10.3% vs. 28.4% ± 7.9%; Figure 5E). However, treatment with amisulpride rescued the pathological memory impairment induced by AAV-eGFP-tau[R406W]. Amisulpride-treated animals spent more time with the less recent object (38.3 ± 3.3 seconds vs. 18.1 ± 2.4 seconds; Figure 5D) and exhibited a higher discrimination index (32.6% ± 8.9% vs. 0.3% ± 10.3%; Figure 5E) than vehicle-treated mice. Importantly, though the level of tau mRNA was selectively increased in PFC after AAV-eGFP-tau[R406W] injection, the expression of 5-HT7R was similar in the three groups (Figures S5D,E).

Though pronounced tau phosphorylation at T205 epitope was observed in GFP-positive neurons at the injection site of mice treated with vehicle, the phospho-tau signal was reduced in amisulpride-treated animals (Figure 5F,G). We also analyzed the extent of neuroinflammation using Iba1-specific antibody as a specific marker of activated microglia. These experiments revealed only weakly increased inflammation at the injection site in both control and amisulpride-treated animals, with no significant differences (Figure S5F,G). These data suggest that amisulpride ameliorates the tau[R406W]-induced effects on memory and tau phosphorylation.

3.6 | Oral administration of amisulpride prevents tau-mediated pathology in tau[P301L]-BiFC transgenic mice

To further validate the in vivo efficacy of amisulpride, we investigated a transgenic mouse model of tauopathy, the tau^{P301L}-BiFC mouse.[19] This line was generated by the genetic knock-in of the human tau[P301L] mutant fused either to the N- or C-terminal part of the Venus fluorophore. We used tau^{P301L}-BiFC mice at the age of 9.5 months, when tau aggregation has already started but neither neuronal death nor cognitive impairment are observed, and orally administered vehicle or amisulpride (1 mg/kg) for 9 weeks. Age-matched WT littermates were used as controls (Figure 6A). In the open field test, we observed a decreased locomotor activity and a more anxious behavior of transgenic mice compared to WT mice. However, there was no significant difference between vehicle-treated and amisulpride-treated tau^{P301L}-BiFC animals (Figure 6B). Next, we performed the novel object recognition test (NORT) and calculated the recognition index to characterize the capacity of mice to memorize and distinguish the two objects (RI; Figures 6C,D). While WT mice were able to distinguish the two objects (RI old: 0.39 ± 0.03 vs. new: 0.61 ± 0.03), the vehicle-treated tau^{P301L}-BiFC mice failed (RI old: 0.49 ± 0.03 vs. new: 0.51 ± 0.03). Treatment of transgenic mice with amisulpride significantly improved recognition memory to levels similar to WT mice (RI old: 0.41 ± 0.05 vs. new: 0.59 ± 0.05). Analysis of spatial working memory using the Y-maze test (Figure 6E) revealed that tau^{P301L}-BiFC mice exhibited a significantly decreased alteration ratio compared to WT mice (52.6% ± 1.7% and 69.0% ± 2.2%; Figure 6F) indicating deficits in spatial working memory. More importantly, chronic oral administration of amisulpride rescued the alteration ratio to a value similar to that obtained in WT mice (64.7% ± 2.5%; Figure 6F).

To evaluate whether the beneficial effects of amisulpride on behavior also persist at the cellular level, we analyzed tau aggregation and phosphorylation in brain slices (Figure 6G-I). Tau-BiFC fluorescence was significantly reduced in the motor cortex as well as the hippocampus of amisulpride-treated tau^{P301L}-BiFC mice. Tau phosphorylation assessed by staining with phosphorylation-specific tau antibody recognizing the S202/T205 epitope was significantly decreased after amisulpride treatment in both brain regions of tau^{P301L}-BiFC animals. These findings were further supported by biochemical analysis of soluble and insoluble brain lysate fractions (Figure S6A-D in supporting information). We observed that the amount of tau in the insoluble brain fraction was drastically reduced in amisulpride-treated transgenic animals. Moreover, while the levels of transgenic human as well as endogenous mouse tau in the soluble fraction were not affected by amisulpride, tau phosphorylation at two tau epitopes associated

Representative images of neurons infected with AAV-eGFP-tau[R406W] (green). Arrowheads indicate tangle-positive neurons (see also Figure S2A in supporting information). Scale bar: 100 µm. C, The number of tangle-positive neurons was counted in a confined area and is presented as a fraction of the total number of infected neurons. Data are shown as means + SEM ($4 \leq N \leq 8$). $^{****}P < 0.0001$; n.s., not statistically different compared to H_2O, one-way ANOVA, Dunnett's multiple comparisons test. D, Representative western blot analysis after sarkosyl fractionation. Tau signals in soluble, sarkosyl-soluble, and -insoluble fractions (i.e., tau-aggregates) were detected by GFP antibody. The βIII tubulin expression was detected in the soluble fraction. E, Apoptotic cells showing caspase3/7 activity were counted in a confined area and are represented as a fraction of the total number of infected neurons. Data are shown as means + SEM ($4 \leq N \leq 7$). $^{****}P < 0.0001$; n.s., not statistically different, one-way ANOVA, Dunnett's multiple comparisons test. F, Experimental design in human iPSC-derived neurons. Human neural stem cells (NSCs) with or without tau[R406W] mutation were differentiated into cortical neurons for 35 days, treated with H_2O, 50 µM amisulpride, or 50 µM tiapride for 3 days, and analyzed at day 38. G, Western blot quantification of lysates from human cortical neurons expressing tau[R406W] treated as indicated. Membranes were probed with pT181 tau, pS202/pT205 tau (AT8), total tau, or GAPDH antibody. Signals are normalized to DMSO-treated cells. Data are presented as means + SEM ($3 \leq N \leq 4$). $^{**}P < 0.01$; n.s., not statistically different compared to DMSO, one-way ANOVA, Dunnett's multiple comparisons test. AAV-eGFP-tau, adeno-associated virus–enhanced green fluorescent protein–tau; ANOVA, analysis of variance; DIV, day in vitro; DMSO, dimethyl sulfoxide; FTDP17, frontotemporal dementia with parkinsonism 17; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; iPSC, induced pluripotent stem cells; NSCs, neural stem cells; PFC, prefrontal cortex; SEM, standard error of the mean; WT, wild type.

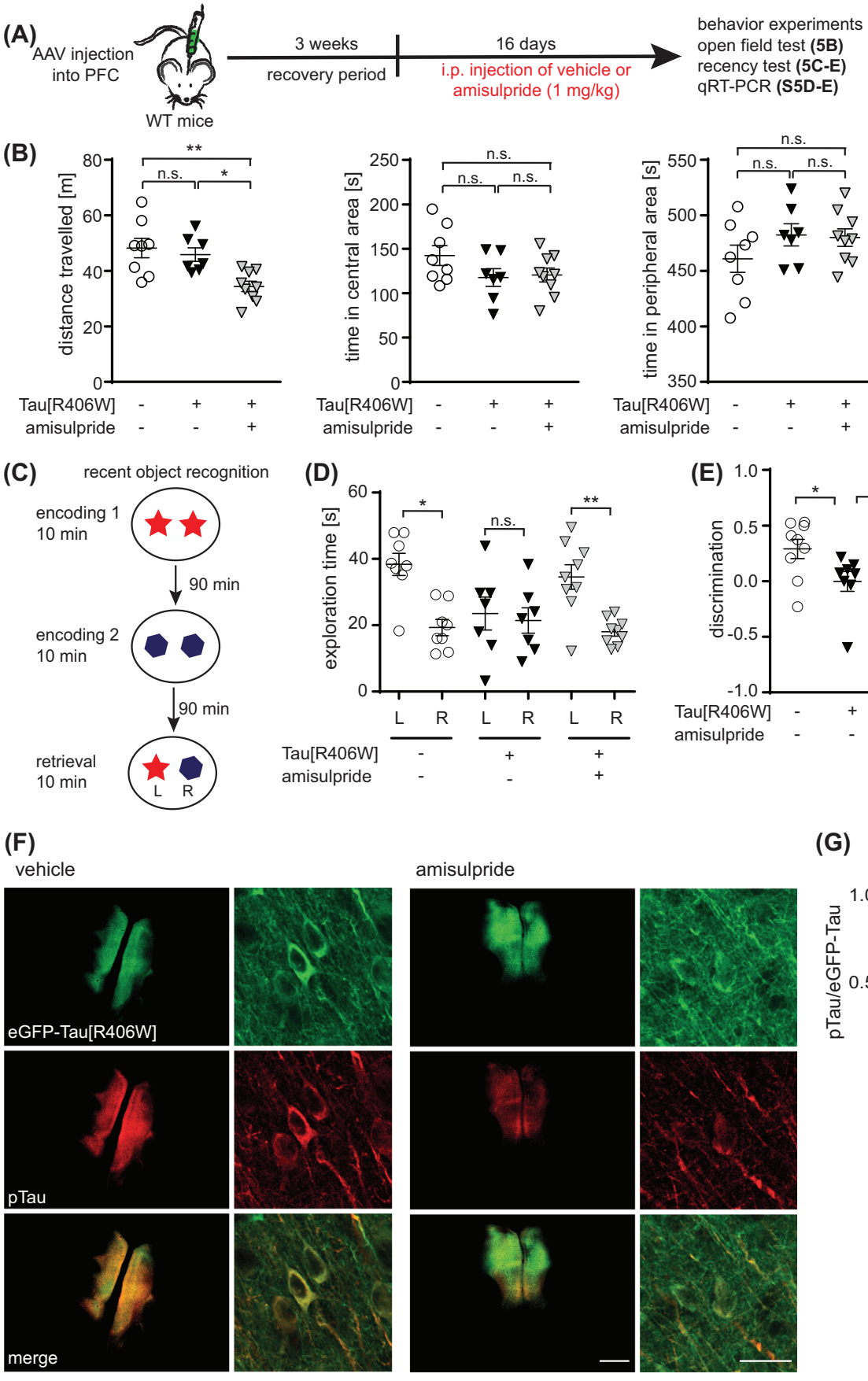

FIGURE 5 Tau-induced memory impairments in mice are ameliorated by amisulpride. A, Experimental design. AAV-eGFP-tau[R406W] or AAV-eGFP were injected stereotactically into the PFC of WT mice. After 3 weeks of recovery time, animals were intraperitoneally injected with either vehicle or amisulpride (1 mg/kg) once daily for 16 days. B, Results of mice performing the open field test after treatment with either vehicle or amisulpride. Quantification of total distance travelled and time spent in the central or peripheral areas. Data are shown as means ± SEM ($7 \leq N$

with the early and advanced transition into tau pre-tangles, S199 and S396,[25,26] was significantly decreased.

These findings demonstrate that the chronic oral administration of amisulpride significantly alleviates the progression of tau pathology in the tau^{P301L}-BiFC mouse model.

4 | DISCUSSION

Decreased serotonergic innervation, reduced activity of the serotonin system, and diminished expression of defined serotonin receptors associated with profound neuronal atrophy have been concomitantly observed in AD and FTD.[27–30] However, the mechanisms connecting serotonergic signaling with AD- and FTD-related neuronal atrophy are not completely understood. Consequently, therapeutic approaches based on modulation of the serotonergic system are currently not available. Two clinical phase III studies using 5-HT1A receptor agonist xaliproden (NCT00104013 and NCT00103649) failed to show a clinical benefit, but phase III clinical trials with 5-HT6 antagonist intepirdine are currently being carried out (NCT02585934 and NCT02586909). However, no trial has addressed the role of 5-HTRs in the treatment of tau-mediated pathology. We recently discovered novel molecular mechanisms by which 5-HT7R modulates pathological tau aggregation.[9] In the present study, we performed comprehensive verification of 5-HT7R as a novel therapeutic target in well-established in vitro and in vivo preclinical models of AD. In particular, we found that the antipsychotic drug amisulpride possesses a high efficacy to block constitutive 5-HT7R activity and demonstrated its high potential in preventing/dispersing tau aggregation and tau-mediated pathology at cellular and systemic levels.

In addition to its affinity for 5-HT7R, amisulpride also targets dopaminergic receptors that, in turn, may influence tau aggregation.[31–33] To exclude the involvement of dopaminergic signaling, we analyzed the effects of dopamine D2/D3 antagonist tiapride. We did not observe any beneficial effects of tiapride on tau phosphorylation, aggregation, or tangle formation in different cellular models, indicating that the effects of amisulpride are 5-HT7R-specific. We also found that vortioxetine, which is a 5-HT7R antagonist, has no effect on tau aggregation, demonstrating that the inverse agonistic properties of amisulpride are responsible for the beneficial effects on tau aggregation.

Taken together, our study demonstrates a potent and previously unrecognized therapeutic effect of amisulpride as a tauopathy treatment. It would therefore be reasonable to validate the potential of amisulpride in future clinical studies. Amisulpride is broadly used in the treatment of acute and chronic schizophrenia. In contrast to other neuroleptics, amisulpride exhibits no affinity for adrenergic, histaminergic, or cholinergic receptors reducing the risk of unwanted drug interactions and adverse effects like fatigue, cardiovascular effects, and weight gain. Nevertheless, some adverse effects are known to occur in the treatment of schizophrenia with amisulpride, for example, insomnia, hyperkinesia, anxiety, and agitation.[34] Moreover, although the extrapyramidal motor effects of amisulpride are less common compared to typical neuroleptics, they can still be observed in individual patients. Amisulpride also has a sedative effect,[35] which we also detected in WT mice treated with amisulpride.

Therefore, the concentration of amisulpride should be carefully adjusted for treatment of patients with tauopathy. The optimal clinical response of amisulpride in schizophrenia patients was obtained at doses of 400 to 800 mg/day, corresponding to plasma levels of approximately 200 to 500 ng/mL with the extra-pyramidal symptoms appearing with plasma levels above 320 ng/mL.[36] More recent pharmacokinetic studies for treatment of psychosis in older patients with AD revealed an effective amisulpride dose in the range of 25 to 75 mg daily.[37,38] Average steady state blood levels with this dosage were 71 ± 30 ng/mL, and antipsychotic clinical response occurred at a threshold concentration of 20 ng/mL. Given that the amisulpride affinity for 5-HT7R is approximately 3-fold lower than that for the dopamine receptors (Ki for D2/D3 receptors = 4 nM; Ki for 5-HT7R = 11 nM), a daily dose range of 150 mg/day seems to be more appropriate for future clinical studies.

Besides dopamine receptors, amisulpride additionally targets 5-HT2BR, which is critically involved in cardiac and pulmonary tissue function.[39–41] Due to its low abundance in the central nervous system, we did not address possible amisulpride effects on 5-HT2BR in this study. However, drugs targeting the 5-HT2BR have been strongly associated with valvular heart disease;[42] thus, special attention must be paid to amisulpride side effects in patients.

Notably, the commercially available amisulpride is a racemate consisting of R- and S-enantiomers, which possess different binding affinities for 5-HT7R.[43] Pharmacological analysis revealed that R-amisulpride is more potent against 5-HT7R relative to S-amisulpride (K_i 47 vs. 1900 nM, respectively), whereas S-amisulpride is more potent against dopamine receptor D2R (K_i 4.0 vs. 140 nM).[44] This suggests that the clinical application of the R-enantiomer will maximize the effect on 5-HT7R and minimize the D2 receptor–related

≤ 9). *$P < 0.01$; **$P < 0.01$; n.s., not statistically different, one-way ANOVA, Sidak's multiple comparisons test. C, Scheme showing the procedure of temporal order recognition test. D, Quantification of exploration time spent at less recent (L) and recent (R) object. Data are presented as means ± SEM ($7 \leq N \leq 9$). *$P < 0.05$; **$P < 0.01$; n.s., not statistically different, paired *t* test and Wilcoxon signed rank test. E, Discrimination index of temporal order recognition test. Data are presented as means ± SEM ($7 \leq N \leq 9$). *$P < 0.05$, Kruskal–Wallis test with Dunn's multiple comparisons test. F, Representative images of prefrontal cortical slices of mice after injection of AAV encoding eGFP-tau[R406W] human mutant. Slices were stained with phospho-specific tau antibody T205. Scale bar overview: 1 mm. Scale bar magnified view: 20 µm. G, Quantification of the mean intensities of eGFP-tau[R406W] and pTau over the whole slice and calculation of the pTau/eGFP-tau ratio. Data are shown as means ± SEM ($N = 5$). *$P < 0.05$, one-tailed unpaired *t* test. AAV-eGFP-tau, adeno-associated virus–enhanced green fluorescent protein–tau; ANOVA, analysis of variance; PFC, prefrontal cortex; pTau, phosphorylated tau; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean; WT, wild type.

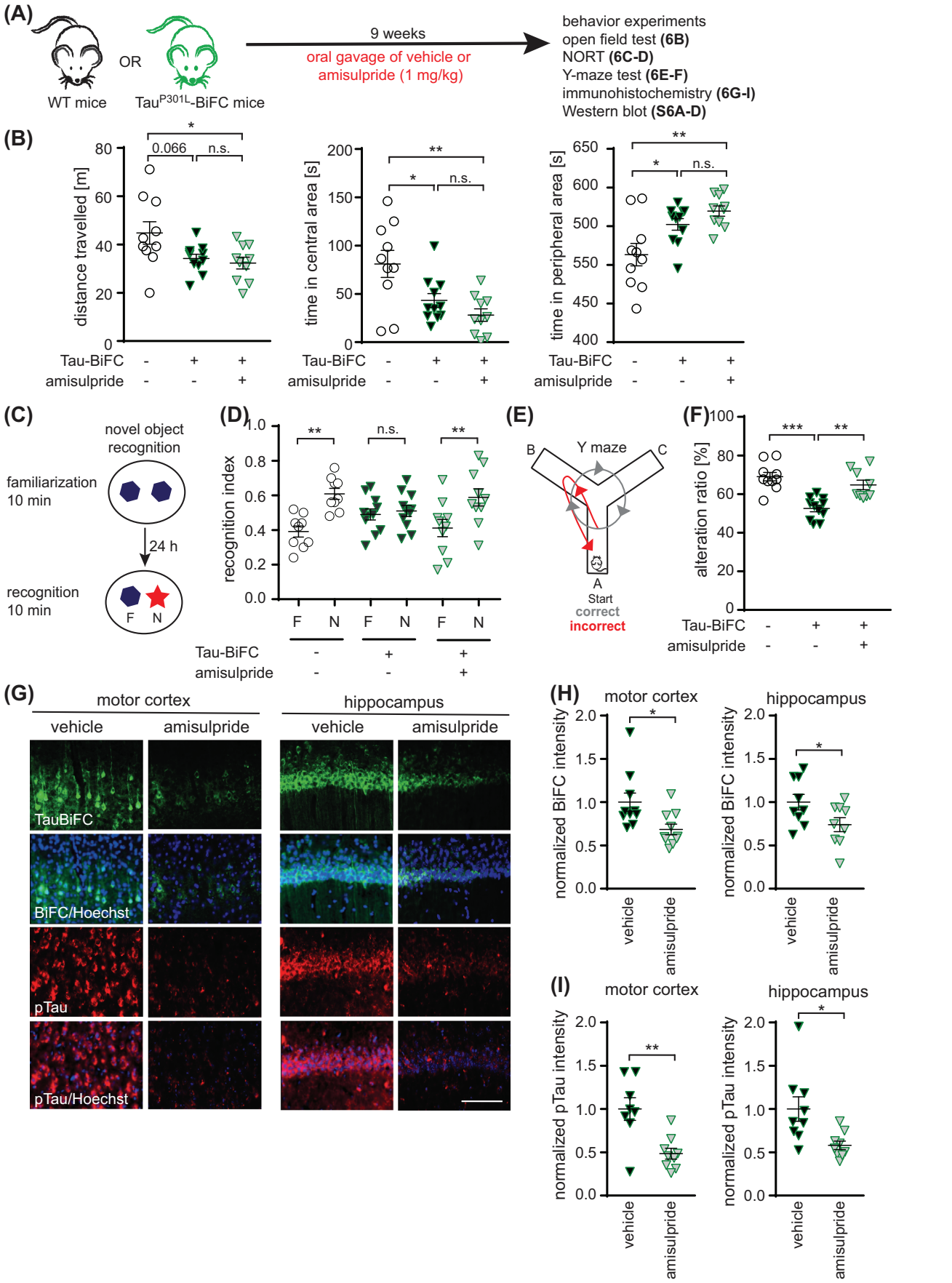

FIGURE 6 Oral treatment with amisulpride alleviates tau pathology in tau^{P301L}-BiFC mice. A, Experimental design. Transgenic tau^{P301L}-BiFC (tau-BiFC) or WT mice were treated orally with either vehicle or amisulpride (1 mg/kg) for 9 weeks. B, Results of the open field test after treatment with either vehicle or amisulpride. Quantification of total distance traveled and time spent in the central or peripheral areas is shown. Data are

extrapyramidal side effects. Therefore, future clinical studies should validate the potential of amisulpride in preventing tau pathology and compare its action to non-racemic amisulpride mixtures or its purified R-enantiomer.

5 | CONCLUSION

In the present study, we demonstrated that several clinically approved drugs including clozapine, amisulpride, and lurasidone possess inverse agonist properties toward 5-HT7R. The detailed pharmacological analysis revealed that amisulpride is the most potent 5-HT7R inverse agonist without cross-reactivity to other serotonin receptors. The therapeutic potential of amisulpride in preventing/dispersing tau aggregation and tau-mediated pathology was validated in vitro (i.e., in HEK293 tau-BiFC cells, human iPSC-derived cortical neurons carrying the tau[R406W] mutation) and in vivo (i.e., in mouse overexpressing of the human tau[R406W] mutant in the PFC and transgenic mouse expressing human tau[P301L] mutant). Using the above-mentioned models of tauopathy, we demonstrated that amisulpride treatment prevents tau hyperphosphorylation, aggregation, and neurotoxicity and abrogates memory impairments in both mouse lines.

Taken together, our results reveal the strong therapeutic effect of 5-HT7R inverse agonists for treatment of tauopathy-related disorders with amisulpride being a more potential disease-modifying drug for tauopathies.

AUTHOR CONTRIBUTIONS

Study design: Josephine Labus, Evgeni Ponimaskin, and Alexander Dityatev. Investigation and analysis: Kathrin Jahreis, Alina Brüge, Saskia Borsdorf, Franziska E. Müller, Weilun Sun, Shaobo Jia, Anastasia Koroleva, Dong Min Kang, Nicolette Boesen, Seulgi Shin, Sungsu Lim, Grzegorz Satała, Andrzej J. Bojarski, Elena Rakuša, Anne Fink, Gabriele Doblhammer-Reiter, Yun Kyung Kim, and Josephine Labus. Writing—original draft: Kathrin Jahreis and Josephine Labus. Writing—reviewing and editing: Weilun Sun, Shaobo Jia, Alexander Dityatev, Evgeni Ponimaskin, and Josephine Labus. Funding acquisition: Yun Kyung Kim, Alexander Dityatev, Evgeni Ponimaskin, and Josephine Labus.

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CONFLICT OF INTEREST STATEMENT

Josephine Labus, Evgeni Ponimaskin, Alexander Dityatev, Shaobo Jia, and Weilun Sun are co-inventors in the international patent WO2020/065090 describing the targeting of 5-HT7 receptor for the treatment of tauopathies. Kathrin Jahreis, Alina Brüge, Saskia Borsdorf, Franziska E. Müller, Dong Min Kang, Nicolette Boesen, Seulgi Shin, Sungsu Lim, Anastasia Koroleva, Grzegorz Satała, Andrzej J. Bojarski, Elena Rakuša, Anne Fink, Gabriele Doblhammer-Reiter, and Yun Kyung Kim do not have any conflicts to declare. Author disclosures are available in the Supporting Information.

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CONSENT STATEMENT

The consent statement is not necessary for this study.

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shown as means ± SEM ($10 \leq N \leq 11$). *$P < 0.05$; **$P < 0.01$; n.s., not statistically different, one-way ANOVA, Sidak's multiple comparisons test. C, Scheme showing the procedure of NORT. D, Recognition index of NORT is shown. Data are presented as means ± SEM ($10 \leq N \leq 11$). **$P < 0.01$, one-way ANOVA, Sidak's multiple comparisons test. E, Scheme showing procedure of Y maze test. F, Alteration ratio of the Y maze test is shown. Data are shown as means ± SEM ($10 \leq N \leq 11$). **$P < 0.01$; ***$P < 0.001$; n.s., Kruskal–Wallis test, Dunn's multiple comparisons test. G, Representative images of brain slices from tau^{P301L}-BiFC mice after vehicle or amisulpride treatment showing tau-BiFC fluorescence (green). Slices were stained with phospho-specific tau antibody pS202/pT205 (AT8, red) and Hoechst (blue). Scale bar overview: 1 µm. H-I, Quantification of mean intensities of tau-BiFC fluorescence (H) and AT8 (I) in the motor cortex and CA1 region of the hippocampus. Data are shown as mean ± SEM normalized to vehicle-treated animals ($8 \leq N \leq 10$). *$P < 0.05$, **$P < 0.01$, two-tailed unpaired *t* test. ANOVA, analysis of variance; NORT, novel object recognition test; SEM, standard error of the mean; tau-BiFC, tau bimolecular fluorescence complementation; WT, wild type.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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