The kainate receptor antagonist UBP310 but not single deletion of GluK1, GluK2, or GluK3 subunits, inhibits MPTP-induced degeneration in the mouse midbrain

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Abstract
The excitatory neurotransmitter glutamate is essential in basal ganglia motor circuits and has long been thought to contribute to cell death and degeneration in Parkinson's disease (PD). While previous research has shown a significant role of NMDA and AMPA receptors in both excitotoxicity and PD, the third class of ionotropic glutamate receptors, kainate receptors, have been less well studied. Given the expression of kainate receptor subunits GluK1-GluK3 in key PD-related brain regions, it has been suggested that GluK1-GluK3 may contribute to excitotoxic cell loss. Therefore the neuroprotective potential of the kainate receptor antagonist UBP310 in animal models of PD was investigated in this study. Stereological quantification revealed administration of UBP310 significantly increased survival of dopaminergic and total neuron populations in the substantia nigra pars compacta in the acute MPTP mouse model of PD. In contrast, UBP310 was unable to rescue MPTP-induced loss of dopamine levels or dopamine transporter expression in the striatum. Furthermore, deletion of GluK1, GluK2 or GluK3 had no effect on MPTP or UBP310-mediated effects across all measures. Interestingly, UBP310 did not attenuate cell loss in the midbrain induced by intrastriatal 6-OHDA toxicity. These results indicate UBP310 provides neuroprotection in the midbrain against MPTP neurotoxicity that is not dependent on specific kainate receptor subunits.

Keywords
Parkinson's disease; UBP310; Kainate receptors; MPTP; Glutamate receptors; 6-OHDA

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[1] 6-OHDA, 6-hydroxydopamine; BG, basal ganglia; DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; i.c.v, intracerebroventricular; KARs, kainate receptors; mGluRs, metabotropic glutamate receptors; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NeuN, neuronal nuclei; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; STN, subthalamic nucleus; TH, tyrosine hydroxylase;

1. Introduction

The progressive neurodegenerative disorder Parkinson's disease (PD) continues to represent a significant health and economic burden. While the use of levodopa remains an effective strategy in the treatment of the motor symptoms of PD, degeneration of the nigrostriatal tract and subsequent loss of dopamine (DA) within the striatum continues to occur. A vast amount of research has therefore focused on identifying the mechanisms underlying this degeneration with the aim of developing novel treatments to halt disease progression.

Excitotoxicity, mediated by glutamate receptors at synapses, has long been thought to contribute to cell death and degeneration in PD due to the critical function of glutamate in the basal ganglia (BG) motor circuit (Blandini et al. 1996, Blandini 2010). Studies initially focused on the ionotropic NMDA and AMPA subtypes of receptors as potential drug targets for PD, as they mediate the majority of fast excitatory transmission in the brain and have long been implicated in excitotoxicity (Klockgether et al. 1989, Koutsilieri et al. 2007, Ambrosi et al. 2014). Despite the evidence antagonists of these receptor subtypes reduce PD pathophysiology in animal models (Loschmann et al. 1991, Turski et al. 1991, Loschmann et al. 1992, Wachtel et al. 1992, Konitsiotis et al. 2000, Blandini et al. 2001), translation of these drugs to the clinic was not similarly successful as they often result in severe adverse side effects such as psychosis, hallucinations, and impaired learning (Lipton 2006, Johnson et al. 2009), likely due to the effects of altering glutamatergic signaling in healthy areas of the brain.

The observation that glutamate is important in PD, together with the failure to translate NMDA and AMPA receptor antagonists has led to a search for alternative approaches to target excitatory neurotransmission. Metabotropic glutamate receptors (mGluRs), have emerged as an alternative target to modifying excitotoxicity as this receptor family plays a modulatory role rather than being involved in fast excitatory neurotransmission (Johnson et al. 2009). As such, mGluR antagonists have been extensively studied as a potential therapeutic alternative in PD (Johnson et al. 2009, Stayte et al. 2014) as targeting them may potentially have less side effects than targeting NMDA or AMPA receptors.

While there has been strong attention on mGluRs for PD, the role of the third class of ionotropic glutamate receptors, kainate receptors (KARs), is less well studied, in part because of a lack of specific pharmacological tools to probe their function. It is known that KARs, like mGluRs, play a modulatory role at synapses, albeit through different mechanisms (Fernandes et al. 2009). Comprised of heteromeric combinations of subunits GluK1-GluK5, KARs are located both postsynaptically where they mediate a component of the excitatory postsynaptic current, and presynaptically where they bi-directionally modify synapse efficacy at both excitatory and inhibitory synapses (Mulle et al. 1998, Contractor et al. 2000, Vissel et al. 2001, Jin et al. 2007). There are currently limited studies of KARs in human or animal models of PD, however expression of these receptors has been demonstrated in both the mouse (Bischoff et al. 1997) and rat brain (Wisden et al. 1993, Bahn et al. 1994, Ghasemzadeh et al. 1996, Wullner et al. 1997), with varying subunit compositions that often include high proportions of the GluK1-GluK3 subunits. In the mouse brain in particular, GluK1 and GluK3 appear to be highly localized to the substantia nigra pars compacta (SNpc), while GluK2 is abundant in the striatum and subthalamic nucleus (STN). Meanwhile GluK4 and GluK5 display little to no expression in these regions. Following these important studies, it was postulated

that KARs, particularly those containing GluK1-GluK3, may be of potential relevance to PD, given they are located in areas critical to both nigrostriatal degeneration and motor function (Wisden et al. 1993, Bahn et al. 1994, Bischoff et al. 1997, Wullner et al. 1997). Therefore KAR antagonists may provide a more selective way to ameliorate glutamate receptor-mediated degeneration compared to NMDA and AMPA antagonists since KARs are primarily modulatory, less abundant, and less ubiquitous. However, direct investigation of this drug class and the receptors they target, specifically in PD, has been lacking.

More recently, the development of KAR specific drugs has allowed for investigations of KAR functions. One such drug is the willardine derivative UBP310, considered to be a GluK1-selective antagonist with nanomolar affinity (Mayer et al. 2006, Dolman et al. 2007) though it also potently antagonizes recombinant homomeric GluK3 receptors in HEK293 cells and CA3 pyramidal cells of wild type mice and rats (Perrais et al. 2009), and displays some actions on GluK2 in a mouse model of temporal lobe epilepsy (Peret et al. 2014). In this study we provide the first evidence that intracerebroventricular (i.c.v) administration of UBP310 provides significant protection of midbrain neurons from degeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, and provide evidence that this effect is not dependent on specific KAR subunits.

2. Materials and Methods

2.1 Animals

C57BL/6 male mice from Australian BioResources (Moss Vale, Australia) or GluK1$^{-/-}$, GluK2$^{-/-}$, or GluK3$^{-/-}$ male mice and their littermate wildtypes (WTs) were obtained at 11 weeks of age. Mice were housed at a maximum five mice per cage for 1 week, until the study began, at which time mice were housed individually. Animals that did not undergo surgical procedures remained in group housing for the duration of the study. Mice were kept on a 12-hour light/dark cycle and access to food and water *ad libitum*. All animal procedures were performed with the approval of the Garvan Institute and St. Vincent's Hospital Animal Ethics Committee under approval numbers 15/07 and 18/16, in accordance with the Australian National Health and Medical Research Council animal experimentation guidelines and the local Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). All surgery was performed under ketamine/xylazil anesthesia, and all efforts were made to minimize suffering.

2.2 Osmotic micropump implantation

Animals were anesthetized with a mixture of 8.7 mg/ml ketamine (Provet) and 2 mg/ml xylazil (Provet) and placed in a stereotaxic apparatus (Kopf Instruments). Osmotic micropumps (Model 1007D; Alzet) were filled with 0.8834 µg/µl (5.3 µg total daily dose) of UBP310 (Tocris) or vehicle control (10% DMSO) and implanted subcutaneously along the back of the neck. An infusion cannula (PlasticsOne) connected to the micropump was placed in the right lateral ventricle at AP -0.2, ML +1.0, DV -2.8 relative to bregma for all MPTP and drug measurement studies. For all 6-OHDA studies the infusion cannula was placed in the left lateral ventricle at AP -0.2, ML -1.0, DV -2.8 to ensure cannula did not impede subsequent lesioning and the pump was removed 1 week later and the cannula tubing sealed with heat. All stereotaxic coordinates were based on the Paxinos atlas for the mouse brain (Paxinos et al. 2001).

2.3 MPTP administration

The day after pump implantation, animals received subcutaneous injections of 20 mg/kg of MPTP (at 2hr intervals x 4 doses; Sigma Aldrich) or vehicle (saline) and placed on heat pads during the injection period and the following 24h to reduce mortality. All mice were euthanized 8 days after the final administration of MPTP or saline.

2.4 Unilateral 6-OHDA lesioning

The day after pump implantation, animals were anesthetized with a mixture of ketamine and xylazil and placed in a stereotaxic apparatus as described above. Mice were then injected with 2 x 2 µl of 3 mg/ml (total 12 µg) of 6-hydroxydopamine (6-OHDA; Sigma Aldrich) in 0.02% ascorbic acid (sham) in the right sensorimotor striatum at the following coordinates: AP +1.0, ML +2.1, DV -2.9 and AP +0.3, ML +2.3, DV -2.9, relative to bregma and the dural surface as previously described (Stayte et al. 2015) and based on the Paxinos atlas for the mouse brain (Paxinos et al. 2001). 6-OHDA (or 0.02% ascorbic acid control) was injected at a rate of 0.5 µl/min and the syringe left in place for 2 min after each injection to allow for complete diffusion. All mice were euthanized 3 weeks after lesioning.

2.5 Immunohistochemistry

Mice were anesthetized and transcardially perfused with ice-cold phosphate-buffered saline (PBS) and 4% paraformaldehyde. Brains were harvested and processed as described in detail previously (Stayte et al. 2015). Sections were incubated in the following primary antibodies: monoclonal mouse tyrosine hydroxylase (TH 1:1000, Sigma Aldrich cat # T2928), monoclonal mouse neuronal nuclei (NeuN 1:500, Merck cat # MAB377) for 72 hours at 4°C. All sections were then incubated in the respective biotin-labeled secondary antibodies (1:250, Abcam cat # AB6813, ThermoFisher Scientific, Cat # B-2770) overnight at 4°C followed by incubation in avidin-biotin complex (Vector Laboratories) at room temperature for 1 h. TH immunolabeling was detected with 3,3'-Diaminobenzidine (DAB, Abacus) until desired staining achieved. NeuN immunolabeling was detected with DAB intensified with nickel ammonium sulfate and counterstained with polyclonal rabbit anti-TH (1:1000, Merck cat # AB152) that was detected with Nova-Red (Abacus) to outline the substantia nigra region.

2.6 Stereology

Quantification of SNpc cell populations was performed using the optical fractionator method and the use of Stereo Investigator 7 software (MBF Bioscience, USA). Sampling parameters for quantification of TH and NeuN positive cells were used as previously described (Stayte et al. 2015, Stayte et al. 2017).

2.7 Catecholamine analysis

Animals were euthanized by cervical dislocation 8 days after the administration of MPTP or saline, the brains removed and the striatum rapidly dissected out and snap frozen. Striata were analyzed for dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) via HPLC as described previously (Stayte et al. 2015). Catecholamine levels were standardized to protein levels measured via Bradford assay and recorded as ng/µg of protein. All standards for the Bradford assay were prepared in freshly made 0.2M PCA + 0.1% L-cysteine.

2.8 Western blotting

Animals were euthanized by cervical dislocation 8 days after the administration of MPTP or saline, the brains removed and the striatum rapidly dissected out and snap frozen. Striata were sonicated in protease inhibitor cocktail (Sigma Aldrich) diluted at a concentration of 1:1000 in RIPA buffer (Sigma Aldrich) and centrifuged at 16,000g for 15 minutes at 4°C. For all samples, 10 µg was loaded onto 4-12% Bis-Tris gels (ThermoFisher Scientific) and protein was separated by running the gel at 180V for approximately 45 min. Following transfer to PVDF membranes (ThermoFisher Scientific), membranes were blocked in 10% skim milk solution for 1 h at room temperature and then incubated in 0.1% BSA solution containing monoclonal rat dopamine transporter (DAT) antibody (Merck cat # MAB369) at a concentration of 1:1000 and kept at 4°C overnight on an orbital shaker. Following washing in buffer containing 1 x TBS buffer without triton + 0.1% Tween 20 for 1 h, membranes were incubated in 5% skim milk solution containing goat anti-rat HRP secondary antibody (Abcam cat # AB97057) at a concentration of 1:1000 for 1 h at room temperature. Membranes were then visualized using Novex ECL Chemiluminescent Substrate Reagent Kit (ThermoFisher Scientific) and visualized in a Chemidoc MP Imaging System (Bio-Rad). Images were then analyzed using Image Lab software (Bio-Rad). All membranes were then stripped and re-probed with monoclonal rabbit anti-GAPDH (Cell Signaling cat # 2188) at a concentration of 1:1000. Raw values were standardized to GAPDH and then adjusted with the values taken from control mice.

2.9 UBP310 measurements

Animals were euthanized by cervical dislocation 24, 48, 72, or 168 h after UBP310 pump implantation and whole brain harvested and snap frozen. Drug naïve mice acted as controls. 100 mg of freeze-dried and powdered tissue was extracted by sonication for 15 min in 1 ml methanol after spiking with 5 ng of internal standard UBP302 (Tocris). This is a close structural analogue of the analyte molecule and was selected as an affordable internal standard. After extraction the samples were centrifuged at 15,000 rpm for 10 min and the supernatant collected and reduced to dryness in a vacuum centrifuge. For analysis, samples were suspended in 100 µl of 1:1 methanol:0.1% formic acid in water and transferred to sample vials for mass spectrometric interrogation. Prior to LC-MS analysis the mass spectrometer (ThermoFisher Scientific Quantum Access) was tuned for maximum sensitivity using continual syringe infusion of a solution containing 100 ng/ml of the two UBP molecules. After tuning the tube lens was held at 145V, the skimmer at 0V, vaporizer temperature at 200°C, capillary temperature at 250°C, sheath gas flow of 20 arbitrary units (au), auxiliary gas flow of 10 au. Optimal selected reaction monitoring transitions were determined for each compound. UBP302 was detected using 334>183 at 16V with the additional qualifying transitions of 334>316 at 10V and 334>116 at 39V. UBP310 was detected using 354>197 at 20V, with the additional qualifiers at 354>308 at 13V and 354>336 at 12V. 10 µl of resuspended sample was injected using a PAL autosampler system (CTC). Samples were chromatographed on a Waters BEH C18 50 x 2.1mm x 1.9 µm UHPLC column using a gradient of 0.1% formic acid in water against acetonitrile. Solvent was delivered at 400 µl/min using an Accela Pump (ThermoFisher Scientific). The solvent composition was held at 100% A for 0.5 min, ramped to 0% A at 4.5 min, held at 0% A until 5 min and then immediately returned to 100% A and held until 7 min for requilibration. Both compounds eluted at 3 min with excellent peak shape using this program. The system was controlled through XCalibur software. Quantification was performed using an external calibration curve over the range 0-10 ng on column,

corrected using the ISTD. Prior to analysis of treated brain samples the system was established as fit-for-purpose by analyzing recoveries from spiked brain material.

2.10 Striatal MPP^+ measurements

Animals were anesthetized with a ketamine/xylazil mixture 90 min after the final MPTP injection followed by cervical dislocation. The brains were removed and the striatum rapidly dissected and snap frozen. Accurately weighed tissue was resuspended at 30 mg/ml in 12% acetic acid in MilliQ water. Samples were extracted by probe sonication for 30 s followed by vortex mixing. After extraction the samples were centrifuged at 15,000 rpm for 10 min and the supernatant collected, diluted 10 times in extraction solvent and transferred to plastic sample vials for mass spectrometric interrogation. Prior to LC-MS analysis the mass spectrometer (ThermoFisher Scientific Quantum Access) was tuned for maximum sensitivity using continual syringe infusion of a solution containing 100 ng/ml of the MPP^+ standard (Sigma Aldrich cat # D048). After tuning the tube lens was held at 83V, the skimmer at 0V, vaporizer temperature at 200°C, capillary temperature at 250°C, sheath gas flow of 20 arbitrary units (au), auxiliary gas flow of 10 au. Optimal selected reaction monitoring transitions were determined – MPP^+ was detected using 170>128 at 33V with the additional qualifying transitions of 170>127 at 33V and 170>77 at 47V. 20 µl of sample extract was injected using a PAL autosampler system (CTC). Samples were chromatographed on a Thermo Acclaim C30 100 x 2.1mm x 3µm UHPLC column using a gradient of 0.1% formic acid in water against acetonitrile. Solvent was delivered at 400 µl/min using an Accela Pump (ThermoFisher Scientific). The solvent composition was held at 100% A for 0.5 min, ramped to 0% A at 4.5 min, held at 0% A until 5 min and then immediately returned to 100% A and held until 7 min for requilibration. MPP^+ eluted at around 3 min with excellent peak shape using this program. The system was controlled through XCalibur software. Quantification was performed using an 8 point external calibration curve over the range 0-500 pg on column. Prior to analysis of treated brain samples, the system was established as fit-for-purpose by analyzing recoveries from spiked drug naïve brain material.

2.11 Statistical analysis

All data were expressed as mean ± SEM. All statistical analysis was performed using GraphPad Prism Version 6.0 (GraphPad Software, Inc). Difference between means was assessed, as appropriate, by unpaired t test or one or two way ANOVA followed by Tukey or Bonferroni post hoc analysis.

3. Results

3.1 Osmotic i.c.v. administration results in measurable quantities of UBP310 in the brain

Investigating the potential neuroprotective effects of UBP310 required administering the drug via osmotic micropumps attached to cannula implanted into the ventricles. To ensure this delivery method resulted in availability of UBP310 to the brain parenchyma, whole brain tissue was harvested at 24, 48, 72, or 168 h after pump implantation. One-way ANOVA of the LC-MS analysis revealed a significant effect of time on UBP310 levels ($F_{(4,30)}$=5.078, p=0.003). Tukey's post hoc tests revealed a significant increase in UBP310 levels compared to drug naïve mice at 24 (p=0.0039), 48 (p=0.0091), 72 (p=0.0375) and 168 h (p=0.0066) however no difference between drug treated animals at any timepoint was observed (Figure 1), indicating i.c.v. administration was sufficient to deliver measurable quantities of UBP310 that were unchanged across time.

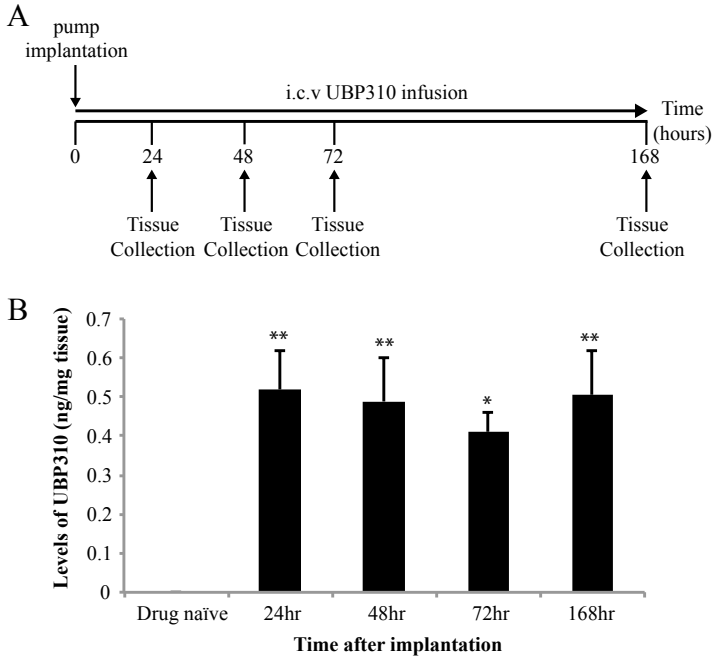

Figure 1. UBP310 levels following intracerebroventricular administration. (A) Experimental timeline. (B) LC-MS analysis of UBP310 levels in whole brain tissue revealed measureable quantities of UBP310 that was unchanged over time. All values represent mean ± SEM. *p<0.05, **p<0.01 (compared to drug naïve mice). N = 7-8/group.

3.2 UBP310 increases survival of nigral neurons following MPTP

To determine the potential neuroprotective effect of UBP310, the number of TH positive cells in the SNpc region was quantified via stereology as a measurement of dopaminergic neuron survival. As expected, MPTP administration resulted in a significant loss of dopaminergic neurons ($F_{(1,37)}$=9.296, p=0.0042), as animals receiving vehicle (10% DMSO) displayed fewer TH positive cells following MPTP compared to their saline controls (p=0.0097 Figure 2B and 2C). However, a significant effect of drug treatment was found ($F_{(1,37)}$=4.953, p=0.0322) with i.c.v. infusion of UBP310 significantly attenuating this effect, increasing the number of surviving TH positive neurons against MPTP-induced toxicity (p=0.0202), without altering baseline numbers in saline treated animals (p>0.9999). Furthermore, the number of dopaminergic neurons in animals receiving UBP310 and MPTP was comparable to those receiving UBP310 and saline (p=0.4669), suggesting a potent neuroprotective effect of UBP310.

To confirm the increased number of surviving TH positive cells with UBP310 treatment was not due to changes in TH expression, as this phenotypic marker can decrease during times of cell stress in the absence of cell death, the number of total neurons in the SNpc was also quantified (Figure 2D). Administration of MPTP significantly decreased the population of total neurons ($F_{(1,35)}$=4.685; p=0.0373), with animals receiving vehicle displaying a reduction in NeuN positive cells following MPTP compared to their saline controls (p=0.0227), however UBP310 was able to attenuate this loss (p=0.0024) without altering the baseline number of total neurons in the SNpc. Furthermore, the number of NeuN positive cells did not differ in animals receiving UBP310 and lesioned

with MPTP, compared to their saline controls. Combined, these results indicate a protection of total neuron numbers with UBP310. Additionally, this neuroprotection is not due to UBP310 altering the metabolism of MPTP, with no significant differences found in striatal MPP^+ levels (p=0.5369) between DMSO (378 pg/mg ± 28.95) and UBP310 (346 pg/mg ± 36.15) treated animals.

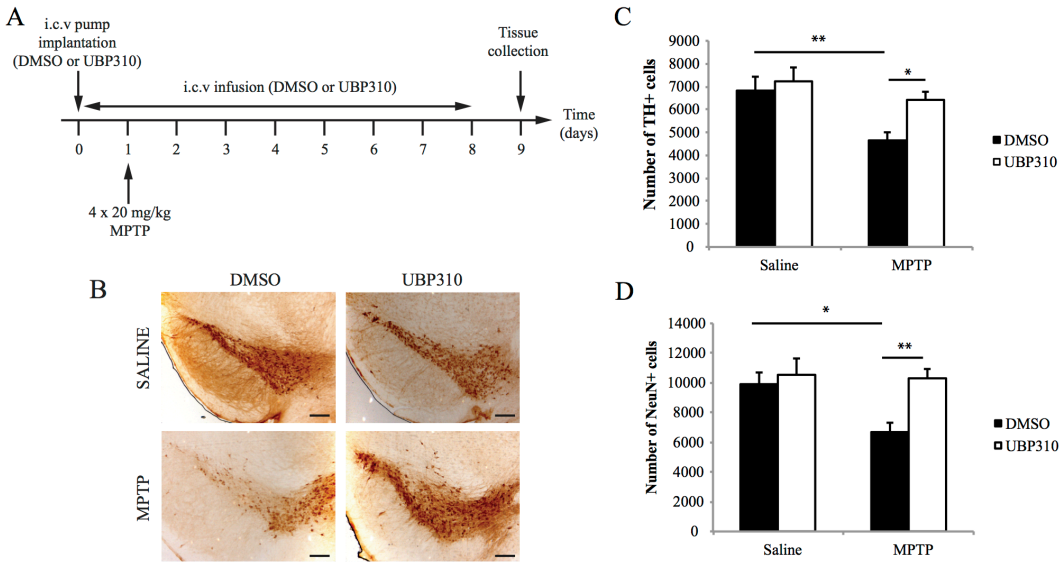

Figure 2. UBP310 is neuroprotective against MPTP-induced toxicity in the SNpc. (A) Experimental timeline. (B) Representative images of TH immunoreactive cells in the SNpc. Stereological analysis in the SNpc 1 week after MPTP administration revealed i.c.v. infusion of UBP310 significantly increases survival of (C) TH positive and (D) NeuN positive cells. All values represent the mean ± SEM. *p<0.05, **p<0.01. Scale bar represents 200 µm. N = 7-13/group

3.3 Effects of UBP310 in the midbrain of KAR subunit knockout mice

While originally identified as a GluK1 specific antagonist (Mayer et al. 2006, Dolman et al. 2007), subsequent studies revealed UBP310 also has actions on GluK2 (Perrais et al. 2009) and GluK3 (Peret et al. 2014). Further, the majority of *in vivo* studies investigating these effects focused primarily on the effects of UBP310 on hippocampal neurons (Pinheiro et al. 2013, Grosenbaugh et al. 2018). Therefore to identify which of these subunits contribute to the previously observed neuroprotective effects of UBP310 in the midbrain, UBP310 was administered to $GluK1^{-/-}$, $GluK2^{-/-}$, or $GluK3^{-/-}$ mice or their littermate WTs in the MPTP model. A cohort of KAR subunit knockout animals were also analyzed in the absence of UBP310 treatment to determine any effect of individual KAR subunits on MPTP-mediated toxicity.

3.3.1 The neuroprotective effects of UBP310 in the midbrain are not altered upon GluK1 subunit deletion

Quantification of TH positive cells revealed no difference between $GluK1^{-/-}$ and WT animals in the saline treated group (p>0.9999), indicating deletion of the GluK1 subunit does not alter baseline levels of dopaminergic neurons in the SNpc. Two-way ANOVA revealed a significant effect of treatment on the number of surviving TH positive neurons in the SNpc ($F_{(2,48)}$=16.59, p<0.0001). Similar to our previous results, UBP310 significantly increased the number of surviving neurons in WT animals following MPTP (p=0.0056), with animals receiving UBP310 displaying cell populations that

were not significantly different from their saline controls (p>0.9999) suggesting a potent neuroprotective effect (Figure 3A). However no significant effect of genotype ($F_{(1,48)}$=0.595, p=0.443) was found, with GluK1$^{-/-}$ mice continuing to display loss of TH positive cells after MPTP (p=0.0013) and subsequent increased survival in the presence of UBP310 (p=0.0100). As shown in Figure 3B, analysis of NeuN positive cells revealed a similar overall effect of treatment ($F_{(2,49)}$=14.19, p<0.0001), with MPTP causing a significant loss of neurons and UBP310 significantly increasing survival in both GluK1$^{-/-}$ and WT animals. No significant difference was found between genotypes within any of the treatment groups ($F_{(1,49)}$=0.05444, p=0.8165). Together these results indicate that deletion of the GluK1 subunit does not alter the neuroprotective effects of UBP310 in the midbrain.

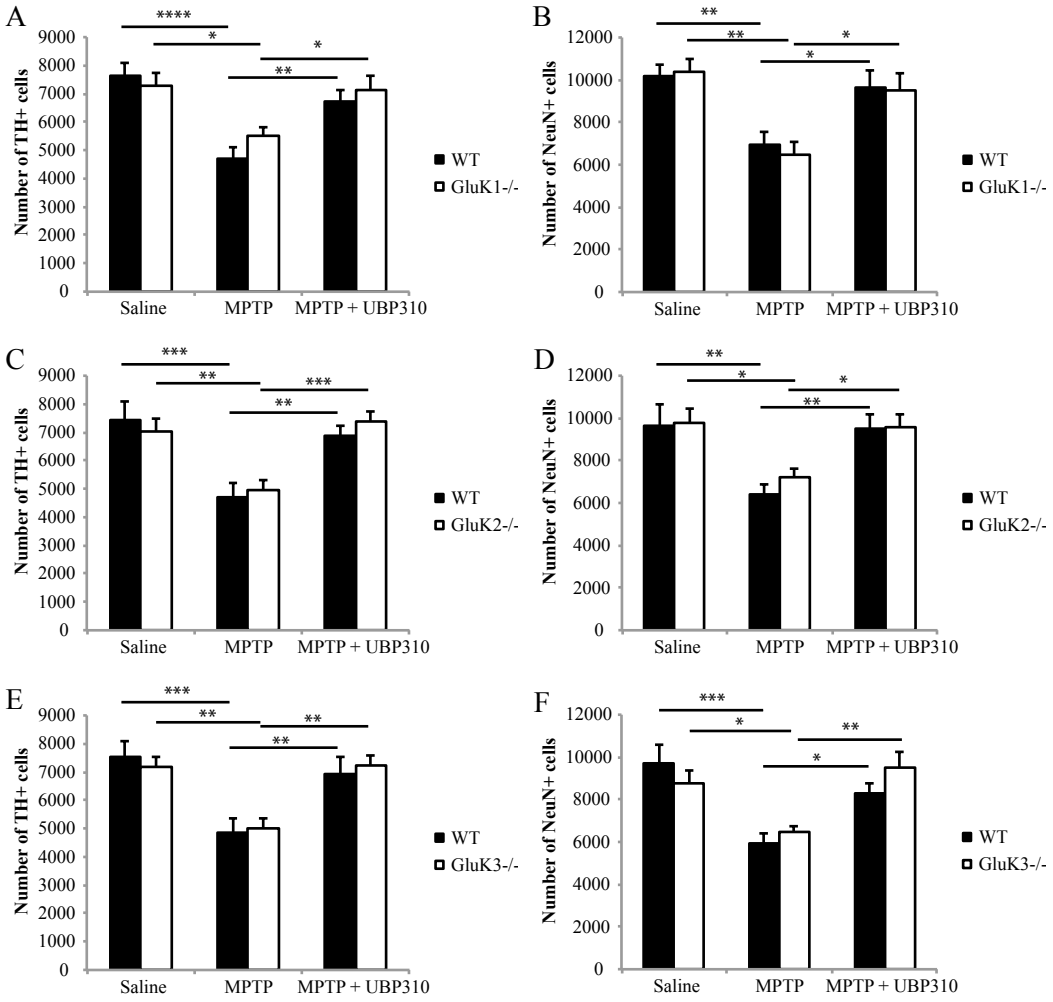

Figure 3. The effect of UBP310 in the SNpc of KAR subunit knockout mice. Stereological analysis of TH immunoreactive and NeuN immunoreactive populations in the SNpc revealed no alterations in the neuroprotective effect of UBP310 against MPTP-induced toxicity in GluK1$^{-/-}$ (A, B), GluK2$^{-/-}$ (C, D), or GluK3$^{-/-}$ (E, F) animals. All values represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N = 8-13/group.

3.3.2 The neuroprotective effects of UBP310 in the midbrain are not altered upon GluK2 subunit deletion

No significant difference in the number of TH positive cell populations between GluK2$^{-/-}$ and WT animals in the saline treated group was found (p>0.9999), demonstrating

deletion of the GluK2 subunit does not alter baseline levels of dopaminergic neurons in the SNpc. An overall effect of treatment on surviving cells was found ($F_{(2,55)}$=12.16, p<0.0001), with both MPTP and UBP310 maintaining their deleterious and neuroprotective effects, respectively (Figure 3C). Post hoc analysis revealed animals treated with UBP310 had numbers of TH cells that were not statistically different from their saline treated controls (GluK2$^{-/-}$ p>0.9999, WT p>0.9999), again suggesting a potent neuroprotective effect of UBP310. Two-way ANOVA with Bonferroni post hoc tests showed no effect of genotype ($F_{(1,55)}$=0.0334, p=0.8557) on TH positive cell numbers, with no significant difference between GluK2$^{-/-}$ and WT animals in both the MPTP (p>0.9999) and UBP310 treated groups (p>0.9999). Quantification of NeuN positive cells yielded similar results (Figure 3D), with UBP310 inhibiting MPTP-induced cell loss ($F_{(2,56)}$=12.42, p<0.0001), an effect that was maintained in both genotypes ($F_{(1,56)}$=0.4053, p=0.5269). Together these results indicate that deletion of the GluK2 subunit does not alter the neuroprotective effects of UBP310 in the midbrain.

3.3.3 The neuroprotective effects of UBP310 in the midbrain are not altered upon GluK3 subunit deletion

No significant differences in the number of TH positive cells between GluK3$^{-/-}$ mice and their littermate WTs in the saline group (p>0.9999) demonstrates deletion of GluK3 does not alter baseline level of dopaminergic neurons in the SNpc. As expected, an overall effect of treatment was found ($F_{(2,53)}$=15.54, p<0.0001) with post hoc analysis confirming MPTP administration resulted in a significant loss of TH positive cells in both GluK3$^{-/-}$ (p=0.0081) and WT animals (p=0.0008). This deficit was rescued with UBP310 administration (GluK3$^{-/-}$ p=0.0058, WT p=0.0118), with UBP310 treated animals displaying cell numbers that were not significantly different from their saline controls (Figure 3E). However no significant effect of genotype was found ($F_{(1,53)}$=0.06284, p=0.8030), with GluK3$^{-/-}$ and WT animals displaying similar cell populations across all treatment groups. As shown in Figure 3F, quantification of NeuN positive cells revealed a similar overall effect of treatment ($F_{(2,49)}$=16.33, p<0.0001), with MPTP causing a significant loss of neurons and UBP310 significantly increasing survival in both GluK3$^{-/-}$ and WT animals while no effect of genotype was found within any of the treatment groups ($F_{(1,49)}$=0.3218, p=0.5731). Together these results indicate that deletion of the GluK3 subunit does not alter the neuroprotective effects of UBP310 in the midbrain.

3.4 Effects of UBP310 in the striatum of KAR subunit knockout mice

3.4.1 Deletion of the GluK1 subunit does not alter MPTP or UBP310-mediated effects in the striatum

Similar to human PD, the loss of dopaminergic neurons with the SNpc in the MPTP mouse model results in a subsequent loss of DA in the striatum, thus a neuroprotection of nigral neurons would likely result in a corresponding protection of striatal DA levels. Therefore striatal tissue was harvested and quantification of DA, DOPAC and HVA via HPLC coupled to an electrochemical detector was conducted. Two-way ANOVA with Bonferroni post hoc analysis revealed MPTP induced a significant loss of DA levels ($F_{(2,17)}$=49.55, p<0.0001) that was unable to be rescued with UBP310 treatment (Figure 4A), contrasting the previous neuroprotective effects in the midbrain. Interestingly, no difference was found between GluK1$^{-/-}$ and WT animals across any of the treatment groups ($F_{(1,17)}$=0.07649, p=0.7854), implicating GluK1 deletion does not alter baseline DA or subsequent MPTP or UBP310-mediated effects. Analysis of DOPAC levels showed a significant interaction ($F_{(2,17)}$=4.722, p=0.0234) between treatment

($F_{(2,17)}$=63.75; p<0.0001) and genotype ($F_{(1,17)}$=1.226; p=0.2837). While GluK1$^{-/-}$ animals had higher baseline levels of DOPAC compared to their WT littermates (p=0.0217), this difference was not maintained following MPTP or UBP310 treatment (Figure 4B). Furthermore, GluK1 deletion had no effect on HVA ($F_{(1,17)}$=0.03876; p=0.8463) or DA/HVA ($F_{(1,17)}$=0.9492; p=0.3436) levels across all 3 treatment groups. Given the important role the DA transporter (DAT) plays in maintaining sufficient DA levels for release into the synaptic cleft, striatal DAT protein levels were also quantified via immunoblotting. Corresponding to the loss of striatal catecholamines, MPTP induced a significant loss of DAT ($F_{(2,47)}$=66.31, p<0.0001) compared to control groups (Figure 4E and 4F), an effect that was not rescued with UBP310 treatment or altered by genotype ($F_{(1,47)}$=0.002879; p=0.957). Together, these results demonstrate GluK1 deletion does not alter MPTP or UBP310-mediated effects in the striatum.

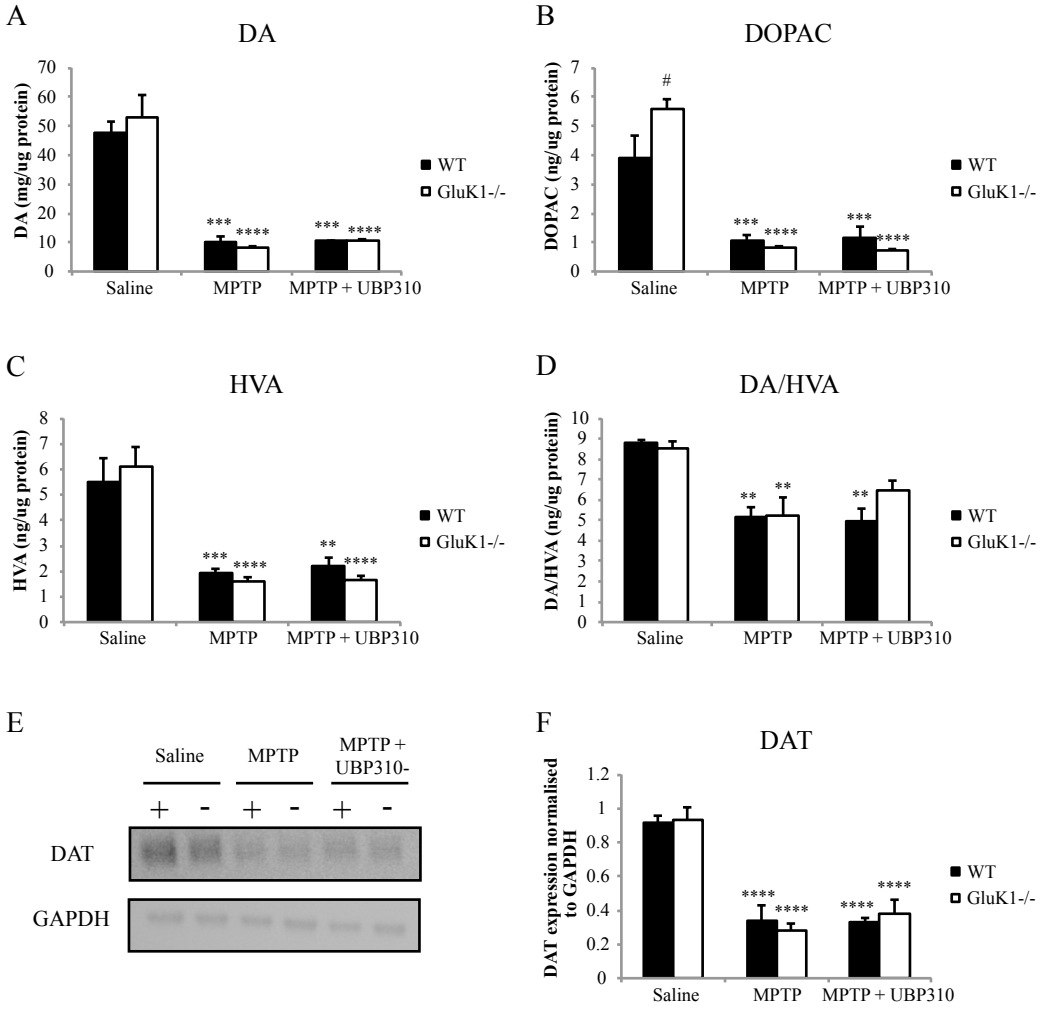

Figure 4. The effect of UBP310 in the striatum of GluK1$^{-/-}$ mice. (A) HPLC quantification of catecholamine levels revealed no effect of UBP310 or GluK1 deletion on striatal DA levels. (B) While GluK1$^{-/-}$ animals had higher baseline DOPAC levels there was no effect of GluK1 deletion on MPTP or UBP310-mediated effects. No effect of UBP310 or GluK1 deletion was found on (C) HVA levels or (D) the ratio of DA to HVA. (E) Representative bands of DAT and GAPDH expression from striatal protein extracts where "+" denotes WT and "-" denotes GluK1 deletion. (F) Quantification of DAT protein expression normalized to GAPDH revealed no effect of UBP310 or GluK1 deletion. All values represent the mean ± SEM. **p<0.01, ***p<0.001,

****p<0.0001 (compared to saline controls), #p<0.05 (compared to WT saline). N = 3-4/group for HPLC analysis, n = 7-10/group for DAT analysis.

3.4.2 Deletion of the GluK2 subunit does not alter MPTP or UBP310-mediated effects in the striatum

No difference in striatal DA levels was found between GluK2$^{-/-}$ and WT animals in the saline treated group, suggesting GluK2 deletion does not affect baseline DA levels. An absence of GluK2-mediated effects on DA levels was further evident in no significant difference between GluK2$^{-/-}$ and WT animals in both the MPTP and UBP310 treated groups (Figure 5A), despite a significant MPTP-induced depletion of DA (Treatment $F_{(2,18)}$=27.94; p<0.0001 Genotype $F_{(1,18)}$=0.07495; p=0.7874). Analysis of the metabolites of DA revealed a significant loss of DOPAC in WT but not GluK2$^{-/-}$ animals following MPTP (Figure 5B), despite a strong trend towards reduction in GluK2$^{-/-}$ mice (p=0.0517). However, a significant loss of DOPAC was found in both WT and GluK2$^{-/-}$ animals treated with UBP310. Similar to results in the GluK1$^{-/-}$ experiments, GluK2 deletion had no effect on HVA ($F_{(1,18)}$=0.1334; p=0.7192 Figure 5C) or DA/HVA ($F_{(1,18)}$=0.5367; p=0.4732 Figure 5D) levels across all 3 treatment groups. MPTP-mediated loss of DAT was also unchanged by both UBP310 treatment ($F_{(2,47)}$=47.55; p<0.0001 Figure 5E and 5F) or genotype ($F_{(1,47)}$=0.9486; p=0.3351). Together, these results demonstrate GluK2 deletion does not alter MPTP or UBP310-mediated effects in the striatum.

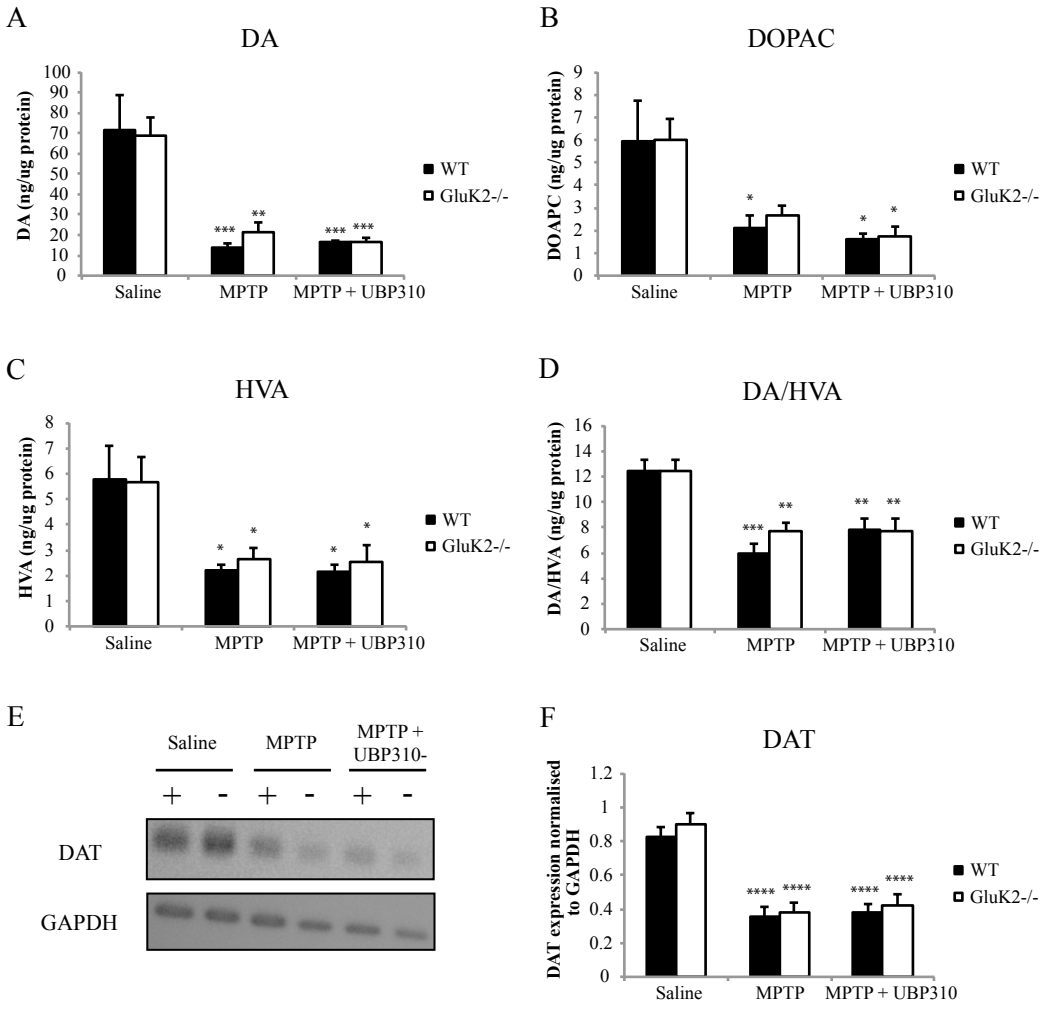

Figure 5. The effect of UBP310 in the striatum of GluK2$^{-/-}$ mice. HPLC quantification of catecholamine levels revealed no effect of UBP310 or GluK2 deletion on striatal (A) DA, (B) DOPAC or (C) HVA levels or (D) the ratio of DA to HVA. (E) Representative bands of DAT and GAPDH expression from striatal protein extracts where "+" denotes WT and "-" denotes GluK2 deletion. (F) Quantification of DAT protein expression normalized to GAPDH revealed no effect of UBP310 or GluK2 deletion. All values represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N = 4/group for HPLC analysis, n = 7-11/group for DAT analysis.

3.4.3 Deletion of the GluK3 subunit does not alter MPTP or UBP310-mediated effects in the striatum

As expected, MPTP administration resulted in a significant reduction of striatal DA levels in both GluK3$^{-/-}$ and WT animals, an effect that was not altered with UBP310 treatment ($F_{(2,17)}$=37.44; p<0.0001 Figure 6A). While there was no difference in baseline levels of DA between both GluK3$^{-/-}$ and WT mice, there was also no effect of GluK3 deletion in both MPTP and UBP310 treated groups ($F_{(1,17)}$=0.8304; p=0.3749). An absence of GluK3-mediated effects was maintained when analyzing DOPAC ($F_{(1,17)}$=2.674; p=0.1204 Figure 6B), HVA ($F_{(1,17)}$=1.763; p=0.2019 Figure 6C) and DA/HVA levels ($F_{(1,17)}$=0.2079; p=0.6542 Figure 6D). Analysis of striatal DAT expression also revealed a similar result, with loss of striatal dopaminergic fibres in both the MPTP and UBP310 treated groups ($F_{(2,53)}$=47.31; p<0.0001 Figure 6E and 6F) that was unchanged between GluK3$^{-/-}$ and WT animals ($F_{(1,53)}$=0.8918; p=0.3493). Together, these results demonstrate GluK3 deletion does not alter MPTP or UBP310-mediated effects in the striatum.

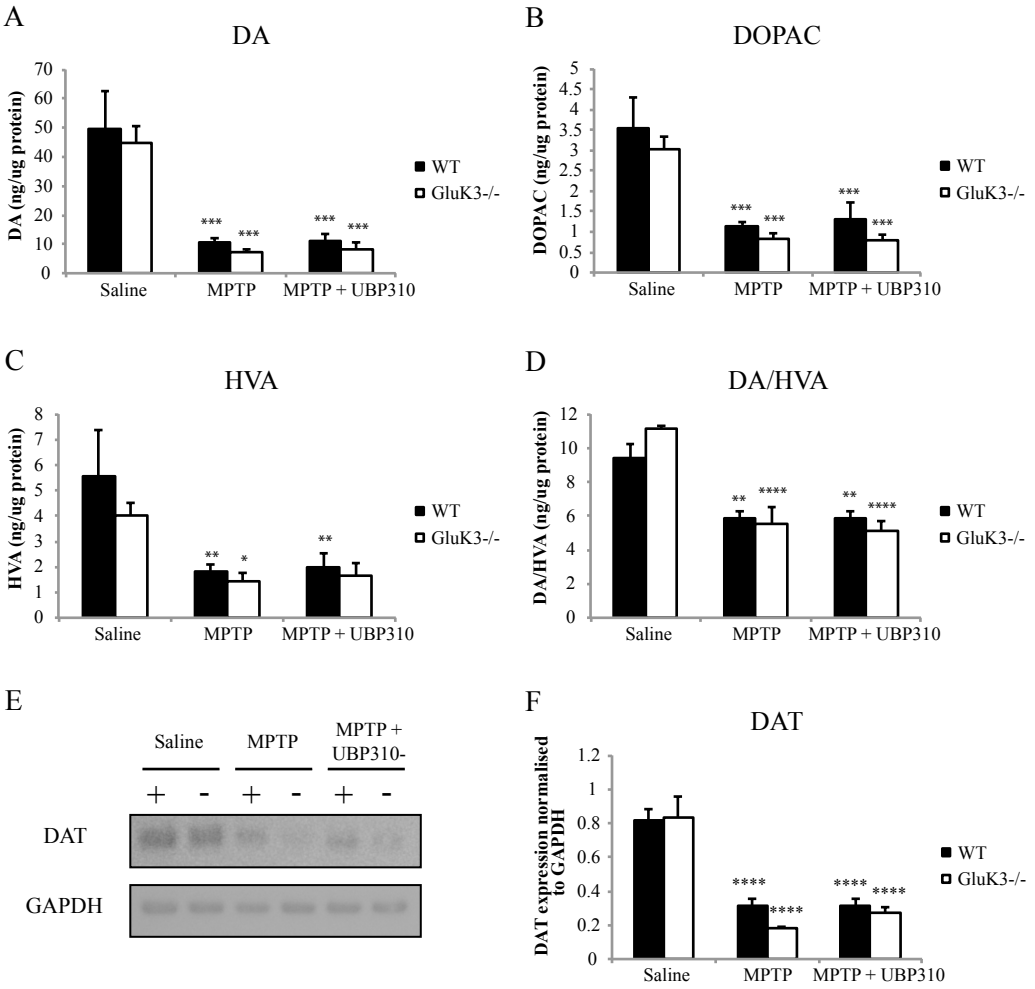

Figure 6. The effect of UBP310 in the striatum of GluK3$^{-/-}$ mice. HPLC quantification of catecholamine levels revealed no effect of UBP310 or GluK3 deletion on striatal (A) DA, (B) DOPAC or (C) HVA levels or (D) the ratio of DA to HVA. (E) Representative bands of DAT and GAPDH expression from striatal protein extracts where "+" denotes WT and "-" denotes GluK3 deletion. (F) Quantification of DAT protein expression normalized to GAPDH revealed no effect of UBP310 or GluK3 deletion. All values represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N = 3-4/group for HPLC analysis, n = 8-13/group for DAT analysis.

3.5 UBP310 is not neuroprotective against 6-OHDA-induced toxicity

To investigate if UBP310 also exerts a neuroprotective effect in the 6-OHDA mouse model of PD, dopaminergic and total neuron numbers were quantified 3 weeks after intrastriatal injection of 6-OHDA. Sham animals receiving UBP310 showed no significant differences in the number of TH positive cells compared to sham animals receiving vehicle (p=0.7882), again demonstrating UBP310 does not alter baseline levels of dopamine neurons in this model. As expected, 6-OHDA resulted in a significant loss of dopaminergic neurons ($F_{(1,24)}$=71.47, p<0.0001), however UBP310 did not protect against this loss ($F_{(1,24)}$=0.1749, p=0.6795), with no significant difference between UBP310 and vehicle treated animals (p=0.3149) in the number of surviving dopaminergic neurons (Figure 7B). Analysis of the number of NeuN positive cells in the SNpc revealed an interaction between toxin and drug treatment ($F_{(1,24)}$=7.598, p=0.110), suggesting a potential effect of UBP310 on 6-OHDA-induced

neuron loss. However Bonferroni post hoc analysis demonstrated no significant difference in the number of NeuN positive cells between UBP310 and vehicle treated animals in both the sham (p=0.1649) and 6-OHDA (p=0.0956) groups (Figure 7C). These results suggest that unlike in the MPTP model of PD, UBP310 is not protective against intrastriatal 6-OHDA-induced degeneration.

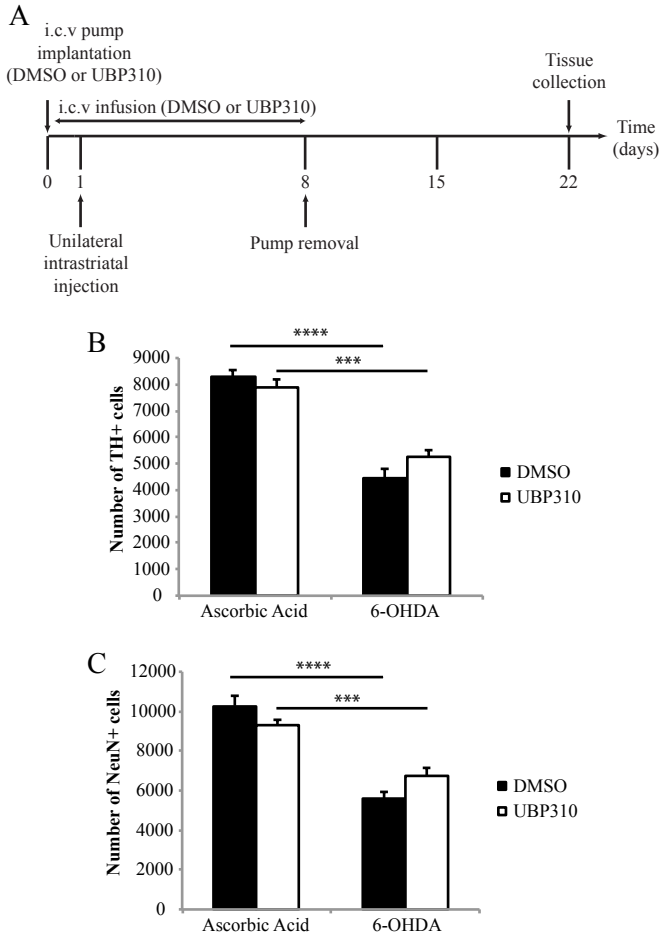

Figure 7. UBP310 does not protect midbrain neurons against 6-OHDA-induced toxicity. (A) Experimental timeline. (B) Stereological analysis of TH immunoreactive neurons in the SNpc 3 weeks after intrastriatal injection of 6-OHDA or ascorbic acid control revealed no effect of UBP310 on dopaminergic neuron populations. (C) Stereological analysis of NeuN immunoreactive neurons in the SNpc 3 weeks after intrastriatal injection of 6-OHDA or ascorbic acid control revealed no effect of UBP310 on dopaminergic neuron populations. All values represent the mean ± SEM. ***p<0.001, ****p<0.0001. N = 7/group

4. Discussion

Owing to its critical role in synaptic transmission within BG motor circuits and evidence of aberrant glutamate signaling in DA-depleted systems, glutamate and its associated receptors have long been linked to the pathophysiology of PD. As a direct result, antagonists against specific glutamate receptor families have been tested as potential therapeutics in both animal models and human clinical trials (Johnson et al. 2009). These studies have largely focused on NMDA and AMPA receptor antagonists, with limited information regarding the contribution of KARs to PD pathophysiology. Research on the neurobiology of KARs has rapidly expanded within the past few decades, driven by the identification and cloning of the receptor subunits and the

development of pharmacological and genetic tools capable of specifically targeting KARs. This has allowed a distinction between the role of AMPA receptors and KARs to be made and led to a growing understanding of the role that KARs not only play in the healthy brain, but in disorders in which abnormalities in glutamatergic neurotransmission are considered to be an important factor, such as PD. The current study is the first to demonstrate that antagonizing KARs is neuroprotective in the midbrain, in an animal model of PD.

Although there is, at present, no perfect preclinical model capable of replicating the entirety of the human PD phenotype, animal models have proven useful for testing novel therapeutic strategies. Both the 6-OHDA and MPTP mouse models employed in this study, for instance, are well-established models for investigating potential neuroprotective agents for PD as they replicate the selective death of dopaminergic neurons within the SNpc and subsequent loss of DA in the striatum. Following demonstration that intracerebroventricular infusion of UBP310 resulted in measurable and stable quantities of the antagonist in the brain parenchyma, the major finding from this work was that UBP310 administration offered significant protection of dopaminergic neuron populations in the SNpc against MPTP-induced toxicity.

Considering its critical role in DA synthesis, TH has long been the standard marker of dopaminergic structures of the brain. However, it is necessary to validate neuron numbers using multiple markers, as TH expression has been illustrated to decrease during times of cell stress, including after MPTP administration (Xu et al. 2005, Kozina et al. 2014). Thus, we corroborated our TH measurements through the quantification of NeuN positive cell numbers, a prototypical marker of neuronal populations. As expected, the numbers of NeuN positive neurons were consistently higher across all groups when compared to the gross number of TH positive cells, which is consistent with NeuN expression in both dopaminergic and non-dopaminergic populations in the SNpc. Importantly however, the significant increase in survival of NeuN positive cells corroborated the increased survival of TH positive cells, providing further evidence that UBP310 was indeed neuroprotective.

KARs consist of tetrameric combinations of subunits GluK1-GluK5, with GluK1-GluK3 able to form functional homomeric or heteromeric receptors, unlike GluK4 and GluK5 which require GluK1-GluK3 to be functional. Multiple in situ hybridization studies have revealed differential expression of individual KAR subunits within the BG of both mice (Bischoff et al. 1997) and rats (Wisden et al. 1993, Bahn et al. 1994, Ghasemzadeh et al. 1996, Wullner et al. 1997), often including high proportions of GluK1-GluK3 compared to GluK4-GluK5. Despite its initial development as a GluK1 specific antagonist (Mayer et al. 2006, Dolman et al. 2007), subsequent *in vitro* and *in vivo* studies provided evidence UBP310 exerts additional actions on GluK2 and GluK3 (Perrais et al. 2009, Peret et al. 2014).

In the current study, global deletion of the GluK1, GluK2, or GluK3 subunit did not alter the survival of dopaminergic or total neuron populations in the SNpc in any of the treatment groups. Both GluK1 and GluK3 have been shown to be highly expressed in the mouse SNpc (Bischoff et al. 1997), likely contributing to the neuroprotective effects of UBP310 through direct inhibition of glutamate-mediated cell death in this region. Injection of the GluK1 agonist ATPA into the SNpc results in ipsilateral turning behavior in rats and subsequent loss of TH positive cells (Vivo et al. 2002), thus it is

interesting GluK1$^{-/-}$ animals did not display neuroprotection against MPTP alone, however this may be due to the differences in GluK1 activation between ATPA and MPTP. While GluK2 has only moderate expression in the SNpc of the mouse, it is highly expressed in the STN (Bischoff et al. 1997). In the presence of aberrant glutamate signaling, as occurs in PD, it is thought increased firing of STN inputs to the SNpc contributes further to DA neuron loss. Thus antagonism of GluK2 in the STN via UBP310 through reducing aberrant BG excitability may contribute to the protection of nigral cell populations, together with direct antagonism of GluK1 and GluK3 containing receptors on DA neurons. Therefore, it remains possible that the neuroprotective effects of UBP310 in the midbrain against MPTP-induced toxicity may result from its actions on all 3 subunits or some combination thereof.

The MPTP mouse model not only induces significant levels of cell death in the SNpc, but also replicates the subsequent loss of DA within the striatum that occurs in PD, an effect that is reproduced in this study. Despite a significant protection of nigral cell populations, UBP310 was unable to attenuate the loss of DA in the striatum, an effect that was not altered in any of the KAR subunit knockout animals. While it could be hypothesized this may be due to UBP310 simply increasing the metabolism of DA, thus reducing the amount of DA present at the time of analysis, this is unlikely as UBP310 also had no effect on the level of the DA metabolites DOPAC and HVA, nor the ratio of DA:HVA. In a study in which MPP^+ was injected into the striatum of rats, followed by various glutamate receptor antagonists, it was found the NDMA antagonists MK-801 and 7-CI-KYN did not lower the DA deficit induced by MPP^+ while the non-selective glutamate antagonist kynurenate and the AMPA/kainate receptor antagonist CNQX both partially attenuated DA loss (Merino et al. 1999). Interestingly, NBQX resulted in a similar protection of DA levels when compared to CNQX and kynurenate (Merino et al. 1999), and given the greater affinity of NBQX for AMPARs over KARs, it is likely protection of striatal DA levels in each treatment group can be attributed to antagonism of AMPARs in this brain region. This is in accordance with the highest levels of AMPAR expression in the BG being found in the striatum (Albin et al. 1992, Ball et al. 1994, Bernard et al. 1996) while there is limited expression of KAR subunits in this area (Bischoff et al. 1997). The lack of UBP310 to provide neuroprotection of nigral neuron populations against 6-OHDA supports this hypothesis, given the striatal injection site of the toxin and the subsequent retrograde degeneration it induces (Blandini et al. 2007, Alvarez-Fischer et al. 2008).

Additionally, UBP310 was unable to attenuate loss of dopaminergic fibres in the striatum in this study, mirroring the loss of DA across treatment groups. While this effect may also be due to the differential expression of KARs in this brain region, the loss of dendrites of nigral neurons has been suggested as an early indicator of neurodegeneration (Bywood et al. 2000) and non-human primate studies utilizing MPP^+ have further supported this idea by suggesting degenerative events are initiated in the striatum (Herkenham et al. 1991). Thus future studies examining the survival of the nigrostriatal tract at a longer timepoint after MPTP, coupled with electrophysiological investigations of the functionality of the surviving neurons, would aid in determining if UBP310 only offers neuroprotection in the midbrain or merely slows the degeneration of the neurons at the level of the soma. In contrast, this discrepancy between midbrain and striatum has been previously shown in studies utilizing MPTP (Liberatore et al. 1999, Feng et al. 2002, Stayte et al. 2017) and

knockout animals (Bodea et al. 2014), suggesting regional-specific effects are indeed possible (Herkenham et al. 1991, Bywood et al. 2000).

The findings from this study raise a number of interesting points. Firstly, the neuroprotective effects of UBP310 appear to be localized to the midbrain, an effect likely due to the combined antagonism of GluK1 and GluK3 within the SNpc itself and potential antagonism of GluK2 within the STN. Direct application of UBP310 to various BG regions rather than via the ventricles as performed in this study would provide further clarification of the subunit-specific mechanisms involved in UBP310's effects. Secondly, previous studies determining the effects of UBP310 on synaptic transmission have been performed primarily on hippocampal neurons (Pinheiro et al. 2013, Grosenbaugh et al. 2018), given the high density of KARS in this region (Mulle et al. 2000, Isaac et al. 2004). Similar electrophysiological experiments conducted in BG neurons and PD models would not only identify if UBP310 alters the functionality of neurons, but how the drug might also modulate the balance between excitatory and inhibitory signaling within the BG and PD, a subject that is likely to be exceptionally interesting. Thirdly, despite detection of specific KAR subunits within the mouse and rat BG (Wisden et al. 1993, Bischoff et al. 1997, Wullner et al. 1997), clarification regarding the expression of GluK1-GluK5 in human BG and any subsequent changes associated with PD remains to be fully understood. Autoradiographic detection of [^{3}H]kainate has revealed in human postmortem tissue with no evidence of disease that KARs are present in PD related brain regions such as the SN, caudate putamen, thalamic nuclei and both the internal and external segments of the globus pallidus (Ball et al. 1994). However limited anatomical resolution of [^{3}H]kainate binding in this study raises the potential that KAR expression can be found in additional BG regions. It also cannot be ruled out that changes to KAR subunit expression may have occurred within the present study in response to select deletion of GluK1-GluK3. As KAR subunits are able to form heteromeric receptors, knockout of a single subunit may not alter the receptor itself as the remaining subunits can form viable KARs. Furthermore, failure of a single subunit deletion to replicate the effect of UBP310 does not rule out the drug working via an action on KARs. Finally, we have primarily focused on the effects of UBP310 on kainate receptors and subsequently, on the glutamatergic system, however future studies could explore other potential mechanisms outside of glutamate signaling as additional drivers of UBP310-mediated neuroprotection.

5. Conclusion

Glutamate-mediated mechanisms have long been thought to contribute to cell death and degeneration in PD, historically leading to the development of NMDA and AMPA receptor antagonists as potential neuroprotective drugs. However, translation of these treatments to the clinic has not always been successful due to the unintentional side effects on fast synaptic neurotransmission in healthy parts of the brain. The findings in this study demonstrating UBP310 has a neuroprotective role against MPTP-induced toxicity in the midbrain suggest that KARs warrant further investigation as an alternative therapeutic strategy for blocking glutamate-mediated cell death in PD.

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Conflicts of Interest
No conflicts of interest exist.

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