Research article

Seizure liability assessments using the hippocampal tissue slice: Comparison of non-clinical species

Michael V. Accardi, Hai Huang, Simon Authier*

CiToxLAB North America, 445 Armand Frappier, Laval, QC H7V 4B3, Canada

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ABSTRACT

Introduction: Traditionally, rat hippocampal tissue slice models are used as an *in vitro* electrophysiology assay to assess seizurogenic potential in early drug development despite non-clinical species-specific differences noted during *in vivo* seizure studies.

Methods: Hippocampal tissue slices were acutely isolated from rats, minipigs, dogs and nonhuman primates (NHP). Population spikes (PS) were evoked through stimulation of the CA3 Schaffer collateral pathway and recorded using *in vitro* electrophysiological techniques *via* an extracellular electrode placed within the CA1 stratum pyramidale cell body layer.

Results: Hippocampal slices, across all species, displayed a concentration-dependent increase in PS area and number with the pro-convulsant pentylenetetrazol (PTZ; 0.1–10 mM). Beagle dogs exhibited higher sensitivities to PTZ-induced changes in PS area and number compared to both rats and NHPs which presented nuanced differences in their responsiveness to PTZ modulation. Minipigs were comparatively resistant to PTZ-induced changes in both PS area and number. Rat and NHP hippocampal tissues were further characterized with the pro-convulsant agents 4-aminopyradine (4-AP; 1–100 µM) and cefazolin (0.001–10 mM). Rats possessed higher sensitivities to 4-AP- and cefazolin-induced changes to both PS area and number whereas NHP displayed greater modulation in PS duration. The anti-convulsant agents, diazepam (10–500 µM) and lidocaine (1–500 µM), were also tested on either rat and/or NHP tissue with both drugs repressing PS activation at high concentrations.

Discussion: Hippocampal tissue slices, across all species, possessed distinct sensitivities to pro- and anti-convulsant agents which may benefit the design of non-clinical seizure liability studies and their associated data interpretation.

1. Introduction

Testing strategies and practices aimed at addressing adverse CNS effects represent an important aspect to the drug development process since they attempt to prevent serious adverse drug reactions from occurring within the clinical setting. These strategies commonly employ behavioral assays such as the Irwin assay (Irwin, 1968) and the functional observational battery (FOB) (Moser, Cheek, & MacPhail, 1995) as well as other more quantitative and objective measures such as electroencephalography (EEG) (Accardi et al., 2016; Authier et al., 2009, 2014; Durmuller, Guillaume, Lacroix, Porsolt, & Moser, 2007). Despite the successful implementation of these testing strategies within safety pharmacology, drug candidates continue to fail within clinical studies due to adverse CNS effects (*e.g.* drug abuse and dependence liability, cognitive impairment, seizures, suicidality) (Bass, Kinter, & Williams, 2004; Hamdam et al., 2013; Harrison, 2016) emphasizing the continued need to minimize drug attrition rates within pharmaceutical

development (Bowes et al., 2012; Kola & Landis, 2004; Pangalos, Schechter, & Hurko, 2007; Waring et al., 2015). Drugs that reach the marketplace are also not without issue, a fact no better exemplified than by Minaprine, an anti-depressive agent, which, in 1996, was withdrawn from the market due to an increase in the clinical incidence of convulsions (Fung et al., 2001). Furthermore, seizures represent one of the most commonly encountered drug-induced preclinical CNS issues found in pre-clinical drug development (Authier et al., 2016). In 2016, 18% of the drugs approved by FDA were for neurology indications, the same proportion as was approved for oncology (Mullard, 2017). CNS drugs tend to be more often associated with CNS adverse events possibly due to higher brain penetration and high affinity for CNS constituents (Kesselheim, Wang, Franklin, & Darrow, 2015). Accordingly, there is growing support for more comprehensive CNS safety testing prior to clinical studies (Lindgren et al., 2008; Valentin & Hammond, 2008) especially when these assays can substitute work using animals.

In vitro brain slice electrophysiological methodologies are

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* Corresponding author.
E-mail address: authiers@ca.citoxlab.com (S. Authier).

commonly employed to address adverse CNS effects within early drug discovery and safety testing (Accardi et al., 2016; Easter et al., 2009; Easter, Sharp, Valentin, & Pollard, 2007). Acute brain slice models, such as the hippocampal tissue slice model, represent a commonly employed *in vitro* electrophysiology preparation since it retains the *in vivo*-like stereotypic and temporal organization and structural integrity of neuronal microcircuits (Gibbs & Edwards, 1994). The hippocampus is recognized as a useful brain slice model due to the fact that it is easily extractable, can remain viable for several hours *ex vivo*, possesses a well-defined lamellar structure for ease of electrode positioning and orientation and represents one of the most well-studied brain regions in neuroscience (Accardi et al., 2016). The hippocampus is also strongly associated with partial (or focal) seizure induction (Schwartzkroin, 1994), an epileptic seizure in which the first clinical and EEG changes indicate initial activation of a system of neurons limited to part of one cerebral hemisphere (Chang, Leung, Ho, & Yung, 2017). Furthermore, the hippocampus plays a central role in temporal lobe epilepsy (Engel, 1996). Taken together, the hippocampus is well suited for use within seizure liability studies. Unsurprisingly, the hippocampal slice model has demonstrated strong concordance to *in vivo* exposure values of various drug candidates and known seizurogenic agents (*e.g.* antibiotics, antidepressants and antipsychotics) (Accardi et al., 2016; Dimpfel, Dalhoff, Hofmann, & Schluter, 1994; Easter et al., 2007, 2009; Fonck, Easter, Pietras, & Bialecki, 2015; Luchins, Oliver, & Wyatt, 1984; Markgraf et al., 2014; Oliver, Luchins, & Wyatt, 1982) with the rat hippocampal tissue slice assay displaying a predictability rate of 89% according to a validation dataset of 19 reference compounds (18 of which were known to induce seizures in man) (Easter et al., 2009). Nevertheless, in spite of this success, *in vitro* brain slice electrophysiology has yet to be consistently successful in predicting toxicities.

Traditionally, *in vitro* hippocampal electrophysiological assays have largely relied upon the rat hippocampal tissue which sometime possesses lower translational value to the human condition (Lynch & Schubert, 1980; Mead et al., 2016; Preuss, 2000). Accordingly, an assessment of hippocampal tissue isolated from several common animal models (*e.g.* rat, dog, minipig and nonhuman primate (NHP)) within a seizure liability assay would provide a better understanding of how differences amongst these species, often noted during *in vivo* seizure liability assessments and drug safety testing, may benefit the design of non-clinical seizure liability studies and their associated data interpretation. To this end, this investigation has begun to address these questions by assessing the performance of common animal model hippocampal tissue within an *in vitro* electrophysiology seizure liability assay using several clinically relevant pro- (pentylenetetrazol (PTZ), cefazolin, 4-aminopyridine (4-AP)) and anti- (diazepam, lidocaine) convulsant agents. In doing so, thisstudy sets out to highlight important differences to consider between non-clinical species as well as the complex nature of drug induced seizure-like activity; important issues within safety pharmacology.

2. Materials and methods

2.1. Statement on use and care of animals

During this investigation, care and use of animals were conducted in accordance with principles outlined in the current Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Resources. CiToxLAB's facility is AAALAC accredited and the procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) prior to conduct. All procedures were conducted as per Standard Operating Procedures (SOPs) in place.

2.2. Animals and housing conditions

All animals were housed under standard laboratory conditions with controlled temperature (21 ± 3 °C), humidity (30%–70%), 12 h light/dark cycle and 10–15 air changes per hour. Temperature and relative humidity were monitored continuously. Sprague-Dawley rats (2–4 months; Charles River Canada, St-Constant, Québec, Canada), cynomolgus monkeys (3–6 years; Guangxi Guidong Primate Development, China and Le Vallon Vieux Grand Port, Mauritius), Beagle dogs (6–14 months; Marshall Bioresources, North Rose, NY, USA) and Göttingen minipigs (6–9 months; Marshall Bioresources, North Rose, NY, USA) were used within this study. The animals were provided a standard certified commercial chow (Envigo Teklad Certified Global Rodent Diet #2018C, Hi-Fiber Primate Diet #7195C, 25% Lab Dog Diet #8727C and Miniswine Diet #7037C, respectively) and municipal tap water (which has been exposed to ultraviolet light and purified by reverse osmosis) *via* water bottles, automatic watering system and/or bowls, *ad libitum*.

2.3. Preparation of hippocampal tissue slices

Rats were anesthetized with isoflurane, *via* an induction chamber, and immediately decapitated using a rodent guillotine. Dogs, cynomolgus monkeys and minipigs were anesthetized with isoflurane followed by intubation and the whole brain was surgically removed. Upon extraction, the whole brain was rapidly submerged for a minimum of 1.5 min in modified oxygenated (95% O2, 5% CO2) ice-cold artificial cerebral spinal fluid (aCSF) solution, which contained (in mM): 124 NaCl, 3 KCl, 26 NaHCO3, 2 CaCl2, 1.3 MgCl2, 10 glucose (pH 7.3 ± 0.1; 300–330 mOsmol/L). One or both of the hippocampi were subsequently isolated and sliced, using a vibrating tissue slicer (Leica VT100S, Leica Instruments), into 300–500 μm thick coronal or parasagittal slices maintained during the slicing procedures in oxygenated, ice-cold aCSF. On average, a total yield of approximately 6–12 slices were obtained per slicing procedure. The slices were transferred to oxygenated aCSF and held at room temperature (20 °C–23 °C) for at least 1 h before recordings were performed.

2.4. Electrophysiology

Slice experiments were performed at near physiological temperatures (33 °C–37 °C) on a Nikon Eclipse FN1 Upright Microscope (Nikon), equipped with differential interference contrast/infrared optics. Extracellular field recordings were performed on the hippocampal tissue slices using a bipolar stimulating electrode (CBBPE75; FHC, Bowdoin, ME, USA) placed in the CA2/3 stratum radiatum and the Schaffer collateral pathway with a borosilicate glass recording microelectrode (GC150F-10, OD 1.5 mm, ID 0.84 mm; Fisher Scientific; 0.5–4 MΩ, filled with 3 M NaCl) positioned within the CA1 cell body layer. The Schaffer collateral pathway was stimulated at 30 s intervals using constant current pulses (approximately 0.03 ms duration) of varying amplitudes such that evoked population spikes (PS) amplitudes were at least 0.3 mV (the mean PS amplitude was 1.45 ± 0.16 mV, n = 36). Recordings were made with a MultiClamp 700B amplifier (Molecular Devices) in current-clamp mode and Clampex 10.5 (Axon Instruments) was used to control the frequency of the voltage stimuli and to record the evoked response at the recording electrode. PS responses were filtered at 1 kHz and digitized at 25 kHz with a Digidata 1440A data acquisition board. The bath was continuously perfused at near physiological temperatures with aCSF at a rate of 1–3 mL/min. Slices were left to equilibrate for at least 20 min prior performing any *in vitro* electrophysiological recording.

A stimulation– and concentration-response relationship was determined for each slice. Each slice was stimulated, initially in the control aCSF solution, at 30 s intervals over the range 0–65 V (in 5 V increments) until a maximal PS amplitude was achieved. The stimulus

intensity was then re-adjusted for use during the concentration-response experimentation so that the PS amplitude was approximately 50–80% the maximal response. The stability of the recording was assessed during the first 10 min after the selection of the stimulus intensity. Each experimental solution was perfused for a minimum of 10 min, or until a steady-state response was observed, and in ascending drug concentrations. A final stimulation-response relationship was repeated at the end of the experiment within the highest drug concentration tested.

2.5. Data and statistical analysis

For each experimental condition, the effect was taken at the end of perfusion period. To generate a representative response for each condition, an average trace was generated from the last five traces of a given condition from which the mean population spike (PS) area and PS number were obtained; represented numerically as mean ± standard error of the mean (SEM) (unless otherwise specified). For each representative response, effects of the test compound were then quantified relative to control and effects expressed as either a percentage or fold-difference of the control. PS area values were quantified off-line as the absolute PS area (area under the curve centered around the 0 mV line). Statistical significance was determined using an ANOVA test followed by a Bonferroni's correction for multiple comparisons. Statistical analyses between two groups were performed using a Student's *t*-test. For each compound, data were obtained from a minimum of at least two animals. Curve fitting and figure preparation of all electrophysiology data were performed with Origin 7.0 (OriginLab), Microsoft Excel, and Clampfit 10.5 (Molecular Devices).

2.6. PTZ convulsion threshold test

Three (3) minipigs were infused intravenously *via* a temporary saphenous catheter with pentylenetetrazol (PTZ), at a dose concentration of 4 mg/mL, dose rate of 40 mg/kg/h and an infusion rate of 10 mL/kg/h until the first occurrence of tonic convulsion. 0.9% Sodium Chloride for Injection USP (Baxter, Deerfield, IL, USA) was used as the diluent. Clinical signs were monitored continuously during the PTZ infusion. As soon as tonic convulsions were present, animals received diazepam (6 mg/kg, IV) to stop the episode. The PTZ dose delivered at the first myoclonic jerk, clonic convulsion and tonic convulsion was determined.

2.7. Pharmacological compounds

PTZ and 4-aminopyridine (4-AP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cefazolin was obtained from SteriMax (SteriMax Inc., Oakville, ON, Canada). Diazepam was obtained from Sandoz Canada (Boucherville, QC, Canada). Lidocaine was obtained from Vetoquinol (Lavaltrie, QC, Canada). Each compound was dissolved in water prior to its experimental use and further diluted in aCSF to reach its desired concentrations.

3. Results

3.1. Effects of PTZ on rat hippocampal tissue

Upon the application of PTZ, rat PS area, number and morphology displayed notable changes in a dose-dependent manner (0.1–10 mM; n = 6). For instance, changes in PS area were observed at 1 mM PTZ (a fold change of 1.15 ± 0.13), reaching statistical significance ($p < 0.05$) at 10 mM (1.38 ± 0.15) PTZ when compared to the control condition (Fig. 1B). The formation of secondary PSs was typically noted as early as 0.1 mM PTZ, becoming increasingly more prominent, with respect to the first PS, at 1 mM (Fig. 1A–C). For example, the average growth of the 2nd PS relative to the 1st PS was approximately

60% at 10 mM PTZ. All recordings displayed an increase in PS numbers (with increasing PTZ concentrations) with half of the recordings possessing PS numbers in excess of two (maximum PS observed: four) routinely at 10 mM PTZ ($p < 0.05$; Fig. 1B). The amplitude of the first PS remained relatively stable despite increases in both PTZ concentration and the secondary PS size. During the stimulation-response experiments, 10 mM PTZ induced evoked responses with greater PS area than the control condition as early as 15 V, reaching statistically significant levels ($p < 0.05$) as early as 25 V (Fig. 1D). This change in PS area was associated with an increase in PS number (Fig. 1E) from 1.5 ± 0.22 in the control condition to 4.0 ± 0.44 in 10 mM PTZ at a 40 V stimulus intensity ($p = 0.002$).

3.2. Effects of PTZ on nonhuman primate hippocampal tissue

Changes in NHP PS area, number and morphology were noted in a dose-dependent manner (0.1–10 mM; n = 5). Changes in PS area were observed as early as 0.1 mM (a fold change of 1.13 ± 0.12) reaching statistical significance ($p < 0.05$) at 10 mM (1.75 ± 0.28) when compared to the control condition (Fig. 2B). Secondary PS formation was noted in only 2/5 recordings with changes observed within these recordings as early as 1 mM PTZ. Interestingly, the associated growth observed in the secondary spike amplitude, with increasing PTZ concentrations, was limited (Fig. 2C) and no additional PS formations were observed at 10 mM PTZ (maximum PS observed: two) (Fig. 2B). During the stimulation-response experiments, 10 mM PTZ induced evoked responses with greater PS area than the control condition as early as 10 V, reaching statistically significant levels ($p < 0.05$) as early as 30 V (Fig. 2D). This change in PS area was associated with an increase in PS number (Fig. 2E) from 1.4 ± 0.24 in the control condition to 2.6 ± 0.24 in 10 mM PTZ at a 40 V stimulus intensity ($p = 0.004$).

3.3. Effects of PTZ on dog hippocampal tissue

Dog PS area, number and morphology showed robust changes to increasing concentrations of PTZ (0.1–10 mM; n = 4). PS area changes were observed as early as 0.1 mM PTZ (a fold change of 1.19 ± 0.06), continuing to increase in a dose-dependent manner reaching statistical significance ($p < 0.05$) at 3 mM (1.83 ± 0.12) and 10 mM (1.95 ± 0.40) PTZ when compared to the control condition (Fig. 3B). The formation of secondary PSs was typically noted at 1 mM PTZ, becoming increasingly more prominent, in both size and number, at 3 and 10 mM PTZ (Fig. 3A–C). In fact, all recordings showed a robust increase in PS number which reached statistical significance ($p < 0.05$) at 3 and 10 mM PTZ (maximum PS observed: five) (Fig. 3B). Concomitant with the increase in both PTZ concentration and secondary PS number and size, the amplitude of the first PS also displayed a trend to larger amplitudes underscoring an overall increase in neuronal reactivity to the evoking stimulus. A similar trend was also observed within the stimulation-response experiments, where 10 mM PTZ induced evoked responses with greater PS area than the control condition as early as 15 V (Fig. 3D). This change in PS area was associated with a doubling in the PS number from 1.25 ± 0.25 in the control condition to 2.5 ± 0.29 in 10 mM PTZ at a 40 V stimulus intensity.

3.4. Effects of PTZ on minipig hippocampal tissue

Minipig PS area, number and morphology showed little to modest changes in response to increasing concentrations of PTZ (0.1–10 mM; n = 4). For instance, no statistically significant changes were observed in PS area with the largest observed change occurring at 10 mM PTZ (a fold change of 1.48 ± 0.21) (Fig. 4B). The formation of secondary PSs was also minimal with only 2/4 recordings showing increases in PS number (maximum PS observed: five) and any observed growth of the secondary PS was limited to only 14% that of the first PS at 10 mM PTZ which also coincided with a small reduction in the 1st PS amplitude
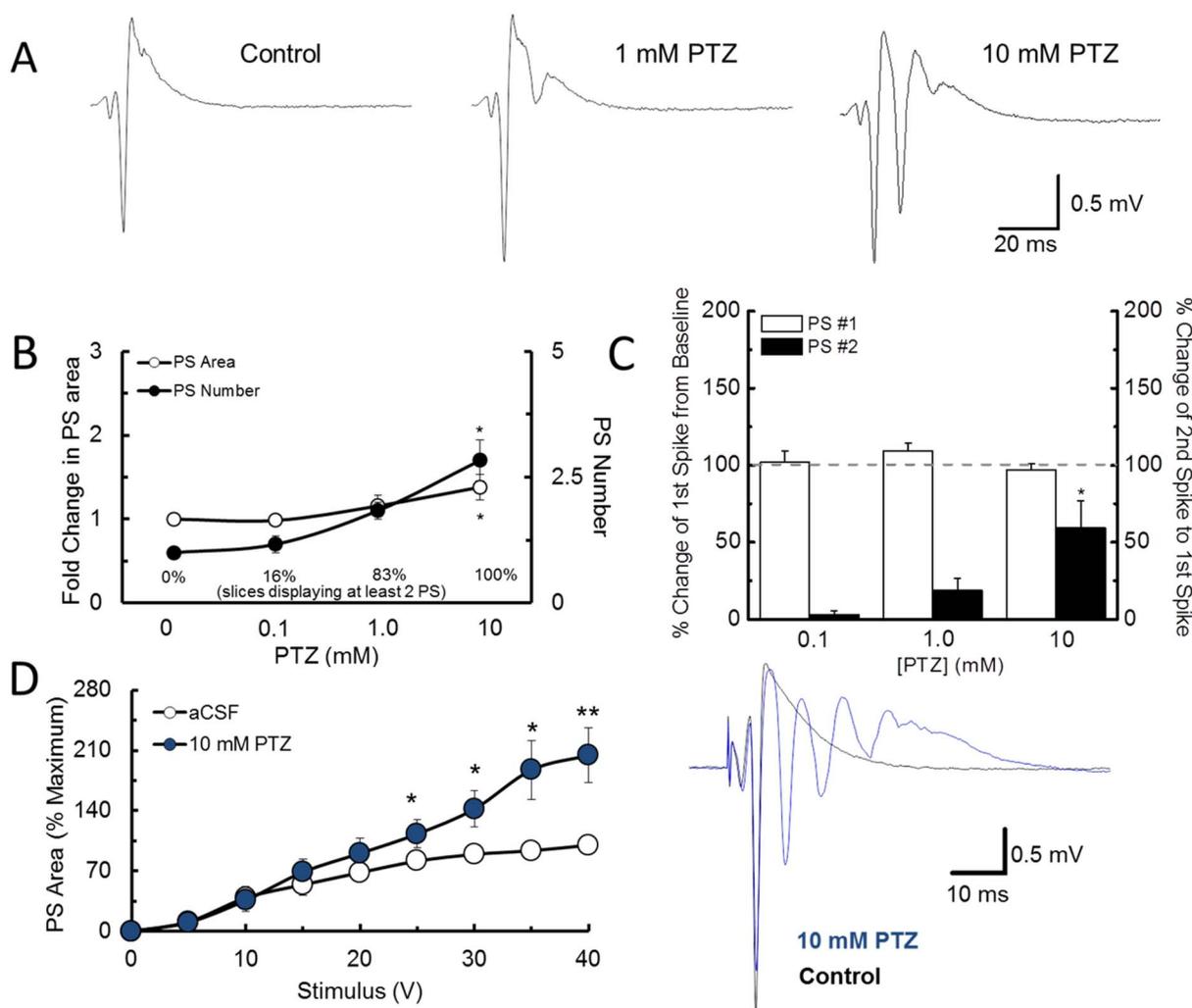
Fig. 1. PTZ-induced changes on CA1 population spikes in rat hippocampal tissue. A) Representative electrophysiological recordings of evoked PS in the absence or presence of ascending PTZ concentrations at a constant stimulation voltage. The stimulus artifact has been removed for clarity. B) PTZ-induced changes to PS area (white circles; left axis) and PS number (black circles; right axis). C) Comparison of the change in PS morphology of 1st PS (white bars; left axis) and the 2nd PS (black bars; right axis). The change in amplitude of the 1st PS has been normalized to the control condition (not shown). The relative amplitude change of the 2nd PS, as compared to the 1st population spike at the given concentration, is shown. D) *Left.* Stimulation-response curve in the absence (white circles) or presence (blue circles) of 10 mM PTZ over increasing stimulus intensities (0-40 V). *Right.* Representative trace of an evoked PS in the absence (black) or presence (blue) of 10 mM PTZ evoked through a stimulus intensity of 40 V. All error bars, ± S.E.M. *p < 0.05; **p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4A–C). The stimulation-response experiments also displayed a modest increase in PS area induced by 10 mM PTZ with greater PS area, compared to the control condition, only reached at stimulus intensities > 35 V (Fig. 4D). In line with these data, *in vivo* susceptibility to PTZ-induced seizures (Table 1) demonstrates that minipigs present relative resistance to seizure induction to a greater extent than all animals tested requiring 69.7 ± 37.4 mg/kg PTZ prior to first clonic convulsion compared to previously published data on dogs (36.1 ± 3.8 mg/kg PTZ), rats (49.4 ± 11.7 mg/kg PTZ) and cynomolgus monkeys (56.1 ± 12.7 mg/kg PTZ) (Bassett et al., 2014).

3.5. Further characterization of rat and nonhuman primate hippocampal tissue

Given the similar PTZ-induced PS changes observed between rats and NHP when compared to the other preclinical species tested, rats and NHP were selected to further evaluate the difference between the two species. To obtain a better characterization of the hippocampal tissue from these species, additional clinically relevant pro- (*e.g.* cefazolin (0.001–10 mM), 4-AP (1–100 μM)) and anti-convulsant (*e.g.* diazepam (10–500 μM), lidocaine (1–500 μM)) agents were applied within

the seizure liability assay. As expected, diazepam induced a reduction in both PS area and number for both rats (n = 3) and NHP (n = 3), almost completely reducing the presence of the PS at 500 μM (Fig. 5A–C). The effects of diazepam were generally first noted at 100 μM to 250 μM through amplitude reductions in the PS, followed by a dramatic reduction in the overall PS area at 500 μM (Fig. 5B and C). The reductions observed for the evoked PSs, in the presence of diazepam, are drug related since control experiments demonstrated PS area and number stability for upwards of 80 min (data not shown) and a wash-period of between 30 and 50 min was able to rescue PS amplitudes and area to pre-diazepam application levels (Fig. 5A).

A similar effect was also observed with lidocaine (n = 2) on NHP. Lidocaine is a local anesthetic that reduces the conductance of the Na$^+$ channel but does not block neuronal transmission at low concentrations (Fried, Amorim, Chambers, Cottrell, & Kass, 1995). Comparable to previous findings in the rat (Fried et al., 1995), NHP tissue showed little to no decrease in PS size up to 100 μM (Normalized to control, PS area: 96.2 ± 0.3%) but was significantly reduced at 500 μM in terms of both PS area (normalized to control: 50.9 ± 3.8%, p = 0.048) and PS amplitude (Control: 1.92 ± 0.09 mV; 500 μM: 0.17 ± 0.03 mV; p = 0.022). As with diazepam, washout (between 15 and 20 min) of
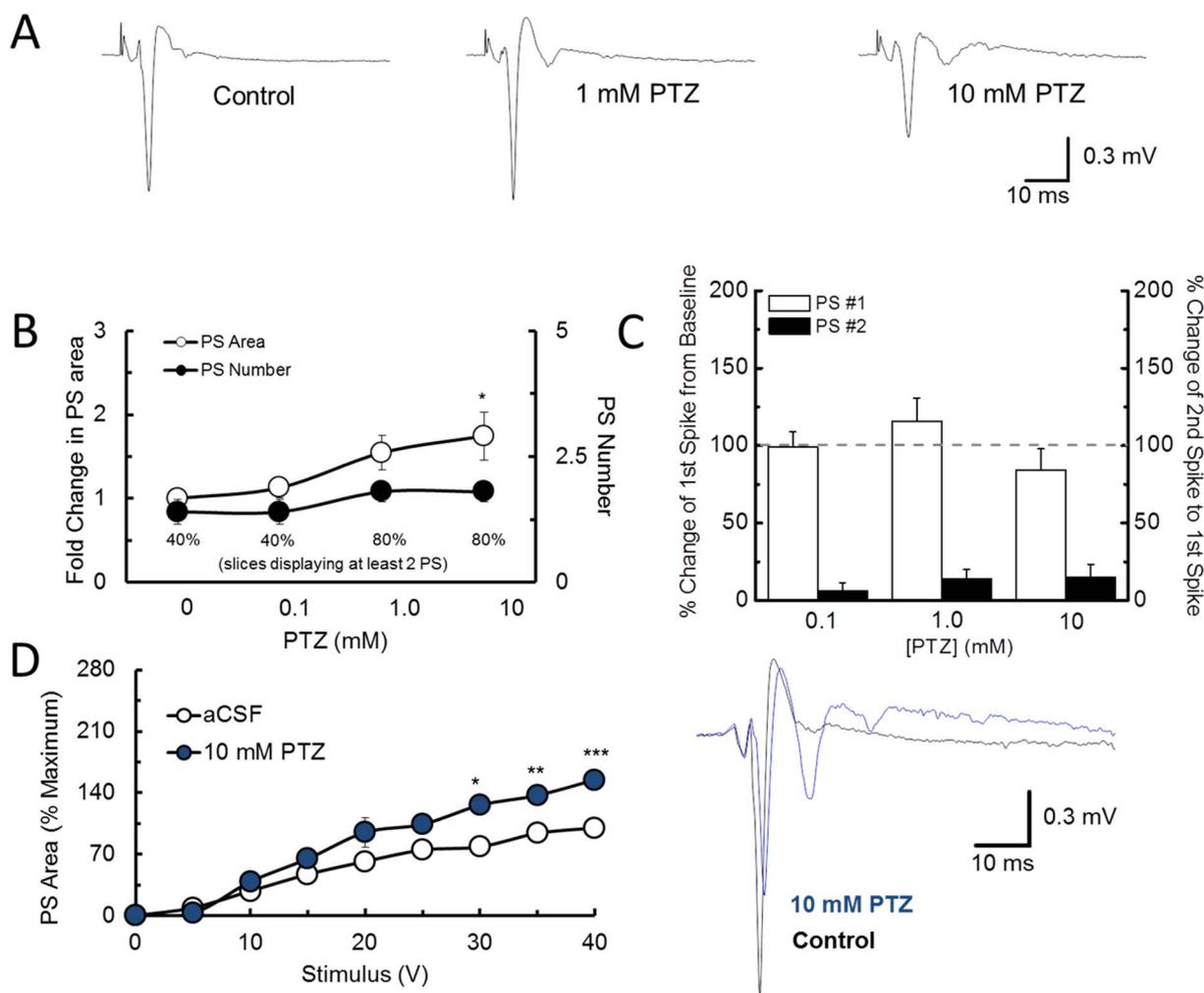
Fig. 2. PTZ-induced changes on CA1 population spikes in NHP hippocampal tissue. A) Representative electrophysiological recordings of evoked PS in the absence or presence of ascending PTZ concentrations at a constant stimulation voltage. B) PTZ-induced changes to PS area (white circles; left axis) and PS number (black circles; right axis). C) Comparison of the change in PS morphology of 1st PS (white bars; left axis) and the 2nd PS (black bars; right axis). The change in amplitude of the 1st PS has been normalized to the control condition (not shown). The relative amplitude change of the 2nd PS, as compared to the 1st population spike at the given concentration, is shown. D) *Left*. Stimulation-response curve in the absence (white circles) or presence (blue circles) of 10 mM PTZ over increasing stimulus intensities (0-40 V). *Right*. Representative trace of an evoked PS in the absence (black) or presence (blue) of 10 mM PTZ evoked through a stimulus intensity of 40 V. All error bars, ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

500 μM lidocaine returned the PS to pre-lidocaine levels (PS area: 112.3 ± 0.02%; PS amplitude: 2.11 ± 0.05 mV).

Applications of 4-AP (rat: n = 3; NHP: n = 4) and cefazolin (rat: n = 4; NHP: n = 2) displayed robust pro-convulsant effects increasing dramatically both PS area and number. For instance, 4-AP induced sizable increases in PS area reaching 6.4- and 4-fold higher levels in rats and NHP, respectively, at 100 μM 4-AP (Fig. 6A, *left*). In addition, rats also displayed a greater increase in PS number compared to NHP with an increase from 1.5 ± 0.5 in the control condition to 5.0 ± 0.7 at 100 μM 4-AP compared to NHP which increased from 1.4 ± 0.24 in the control condition to 3 at 100 μM (Fig. 6A; *middle and right*). Cefazolin also displayed strong pro-convulsant effects with rats presenting a maximum fold increase in PS area of 1.83 ± 0.16 (10 mM) and average increase in PS number from 1 (control) to 4.25 ± 0.85 (10 mM) (Fig. 6B; *left and middle*). Similarly, cefazolin induced pro-convulsant effects within NHP tissue, inducing a maximum fold increase in PS area of 3.29 ± 0.73 (10 mM) (Fig. 6B; *left*). As with 4-AP, cefazolin increased the PS number of NHP from 1 (control) to 4 (10 mM) (Fig. 6B; *middle and right*). Interestingly, despite generally displaying lower evoked PS numbers at the higher dose concentrations for 4-AP and cefazolin, NHP evoked PSs were generally longer in duration compared to rats (Fig. 6B, *middle and right*). For instance,

control rat PS durations were 16.2 ± 0.9 ms and control rat PS durations were 33.5 ± 5.6 ms. In the presence of 4-AP, rat PS durations increased significantly to 56.6 ± 1.9 ms (p = 0.025; 30 μM) and 73.7 ± 12.4 ms (p = 0.037; 100 μM) compared to NHPs which showed significant increases to 87.3 ± 16.1 ms (p = 0.018; 30 μM) and 100.9 ± 14.2 ms (p = 0.0009; 100 μM). A similar observation was observed in the presence of cefazolin with rats and NHP PS durations increasing to 44.7 ± 7.3 ms and 107.4 ± 50.4 ms, respectively, at 10 mM.

4. Discussion

In vitro brain slice electrophysiological assays, such as the hippocampal seizure liability assay, are recognized as part of early safety pharmacology testing strategies (Accardi et al., 2016; Easter et al., 2009). However, despite a relatively high predictability rate of 89% (Easter et al., 2009), the success of rat hippocampal tissue slice assays in safety pharmacology programs may be limited by the differences observed between rodent and human responses (Lynch & Schubert, 1980; Mead et al., 2016; Preuss, 2000). Accordingly, an evaluation of *in vitro* hippocampal tissue slice models in animal species which share closer anatomical and evolutionary ties to humans may represent a potential
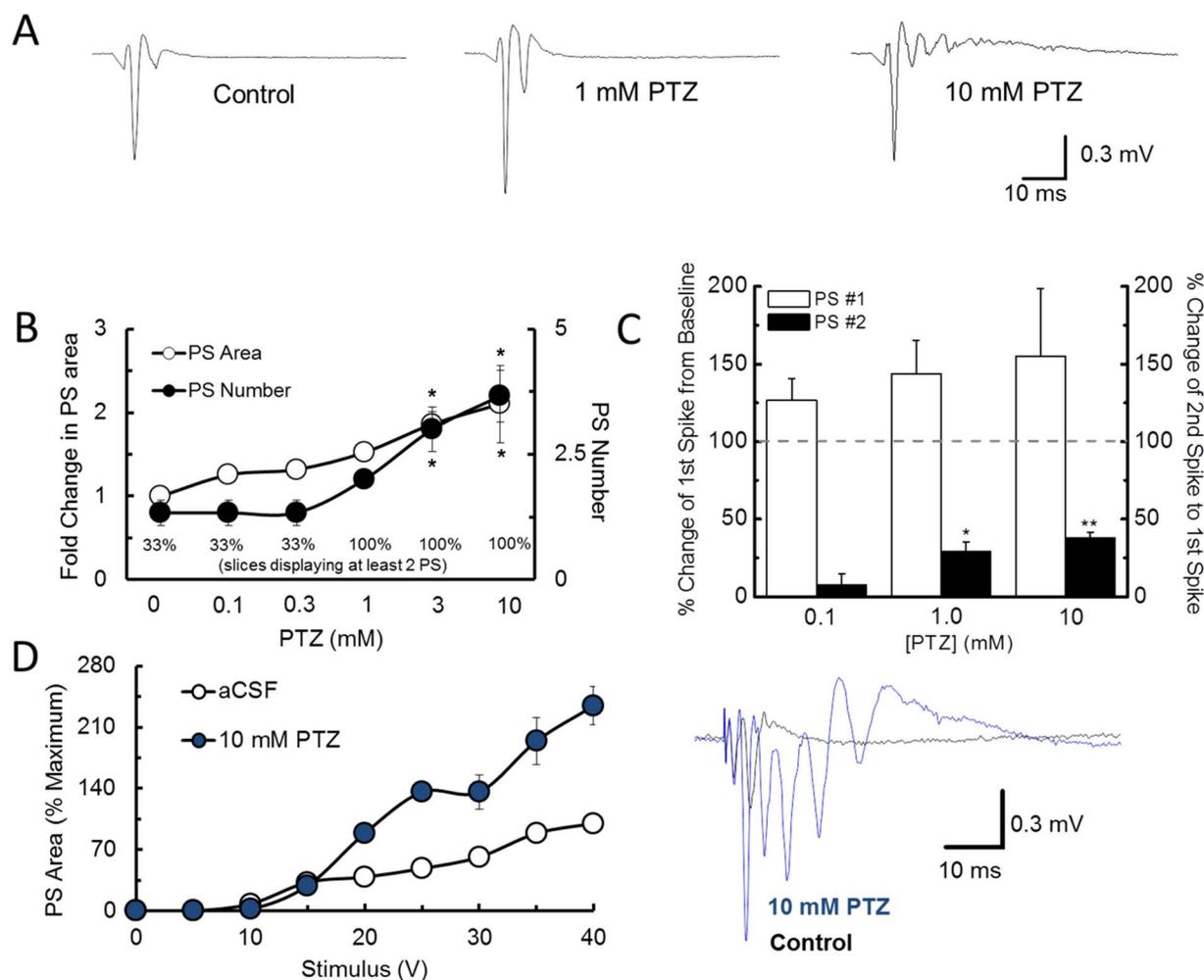
Fig. 3. PTZ-induced changes on CA1 population spikes in dog hippocampal tissue. A) Representative electrophysiological recordings of evoked PS in the absence or presence of ascending PTZ concentrations at a constant stimulation voltage. The stimulus artifact has been removed for clarity. B) PTZ-induced changes to PS area (white circles; left axis) and PS number (black circles; right axis). C) Comparison of the change in PS morphology of 1st PS (white bars; left axis) and the 2nd PS (black bars; right axis). The change in amplitude of the 1st PS has been normalized to the control condition (not shown). The relative amplitude change of the 2nd PS, as compared to the 1st population spike at the given concentration, is shown. D) *Left.* Stimulation-response curve in the absence (white circles) or presence (blue circles) of 10 mM PTZ over increasing stimulus intensities (0-40 V). *Right.* Representative trace of an evoked PS in the absence (black) or presence (blue) of 10 mM PTZ evoked through a stimulus intensity of 55 V. All error bars, ± S.E.M. *p < 0.05; **p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

path to explore translational limitations. To this end, this investigation advances our understanding of the use of several common animal models (*e.g.* rat, dog, minipig and NHPs) within an *in vitro* hippocampal seizure liability assay highlighting the similarities and nuanced differences between each tissue. As a result, a rank order in terms of PTZ-sensitivity can be established which may influence the design of non-clinical seizure liability studies and their associated data interpretation. In addition, the rat and NHP hippocampal tissue was further characterized to assess how these species may predict the seizurogenic potential of clinically relevant pro- and anti-convulsant agents.

One of the most commonly employed pro-convulsant agents used within the clinical arena is PTZ (Wax, 1997). PTZ has long been known to inhibit GABA-activated receptors (Macdonald & Barker, 1978) binding at or near the picrotoxin binding site (Huang et al., 2001). In addition, PTZ has been shown to induce elevations in both neuronal Ca^{2+} and Na^+ levels (Papp, Feher, & Erdelyi, 1987) further promoting pro-convulsant activity within the central nervous system. Thus, due to its role within the clinical setting and wide use as an animal seizure model (Loscher, 1997), we set out to identify the PTZ-sensitivity of hippocampal tissue isolated from several common animal models. In agreement with previously published data which, uncovered an active PTZ concentration range within the *ex-vivo* hippocampal tissue slice of

2–10 mM (Easter et al., 2007; Leweke, Louvel, Rausche, & Heinemann, 1990; Omrani, Fathollahi, Mohajerani, & Semnanian, 2000; Rostampour et al., 2002), this investigation demonstrated an active concentration range between 3 and 10 mM for PTZ within rats, dogs and NHPs. Interestingly, minipigs did not show any statistically significant increases in *in vitro* seizurogenic indicators (*e.g.* PS area and number) (Gutnick, Connors, & Prince, 1982) at the concentration ranges tested. Minipigs have been argued to be a more suitable species for experimental models of diverse human brain disorders than rats since the pig brain, which is gyrencephalic, resembles the human brain more in anatomy, growth and development (Lind et al., 2007). However, as these data demonstrate, some caution may be required for the broad implementation of minipig brain tissue within *in vitro* neuroscience research given the reactivity of the other animal species tested. Nevertheless, minipigs displayed PS morphological changes suggestive of PTZ-sensitivity at higher concentrations. The comparative lack of PTZ-sensitivity observed within minipigs nicely mirrors *in vivo* PTZ data where minipigs displayed a higher threshold for PTZ-induced convulsive behavior (Table 1). It is interesting, however, that a similar delivered PTZ-dose level was observed between Beagle dogs and minipigs for first myoclonic jerk (*i.e.* Minipigs, 24.9 ± 18.8; Beagle dog, 28.8 ± 11.5) despite minipigs possessing a stronger resistance to
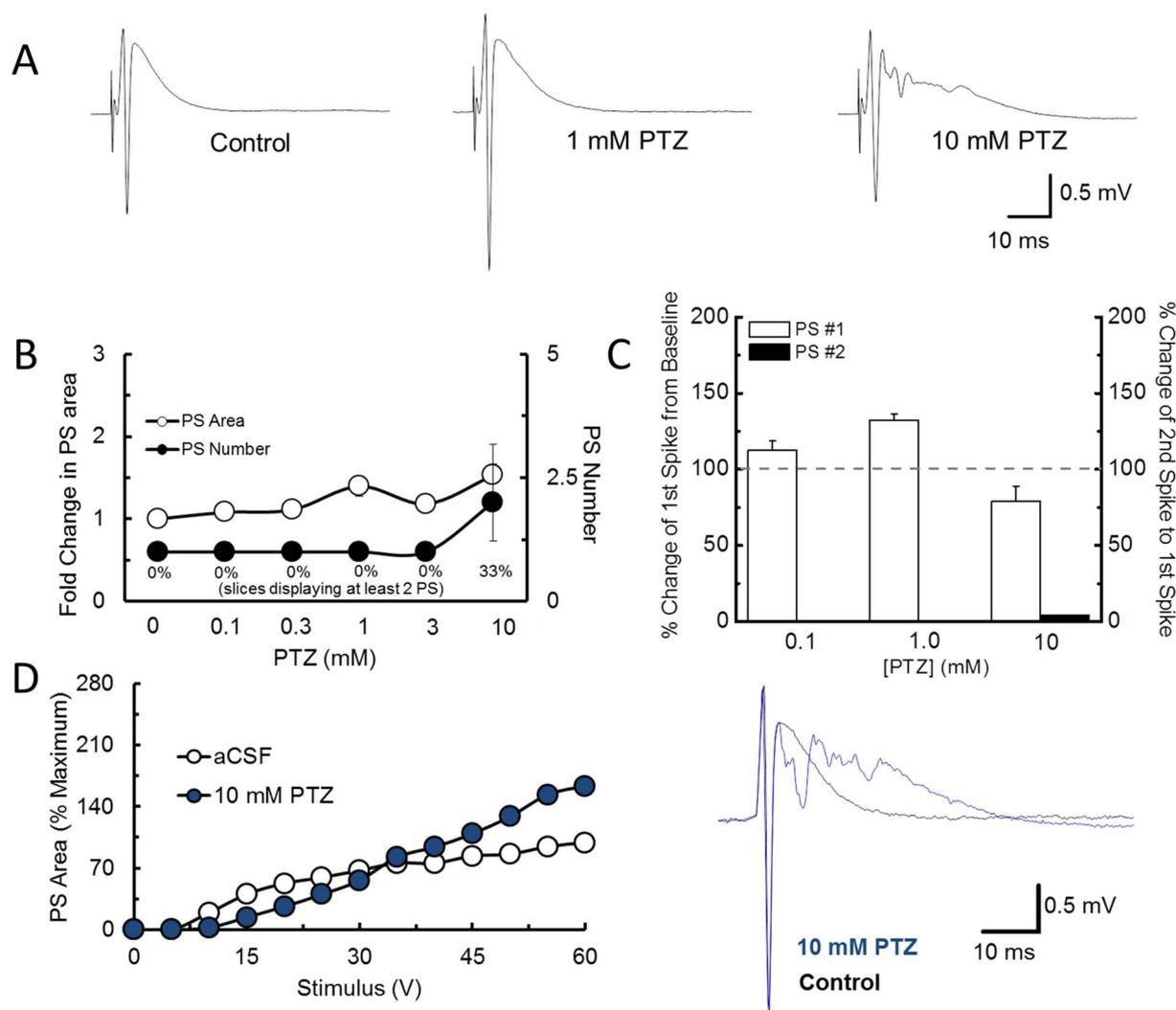
Fig. 4. PTZ-induced changes on CA1 population spikes in minipig hippocampal tissue. A) Representative electrophysiological recordings of evoked PS in the absence or presence of ascending PTZ concentrations at a constant stimulation voltage. B) PTZ-induced changes to PS area (white circles; left axis) and PS number (black circles; right axis). C) Comparison of the change in PS morphology of 1st PS (white bars; left axis) and the 2nd PS (black bars; right axis). The change in amplitude of the 1st PS has been normalized to the control condition (not shown). The relative amplitude change of the 2nd PS, as compared to the 1st population spike at the given concentration, is shown. D) *Left.* Stimulation-response curve in the absence (white circles) or presence (blue circles) of 10 mM PTZ over increasing stimulus intensities (0-60 V). *Right.* Representative trace of an evoked PS in the absence (black) or presence (blue) of 10 mM PTZ evoked through a stimulus intensity of 65 V. All error bars, ± S.E.M. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
In vivo susceptibility to PTZ-induced seizures across multiple preclinical species.

Animal	PTZ Dose (mg/kg)		
	First myoclonic jerk	First clonic convulsion	First tonic convulsion
Beagle dogs[a] (n = 5)	28.8 ± 11.5	36.1 ± 3.8	36.8 ± 5.4
Cynomolgus monkeys[a] (n = 12)	40.5 ± 14.2	56.1 ± 12.7	57.3[b]
Sprague Dawley rats[a] (n = 24)	43.8 ± 5.5	49.4 ± 11.7	93.3 ± 13.3
Göttingen minipigs (n = 3)	24.9 ± 18.8	69.7 ± 37.4	83.7 ± 28.7

All Data is represented as 'mean ± standard deviation'.
[a] Data obtained from Bassett et al. (2014).
[b] Clonic convulsions were treated in cynomolgus monkeys and only one animal presented tonic convulsions.

PTZ-induced seizure activity. This may be explained by the fact that some myoclonic jerks have been known to originate in non-cortical regions (*i.e.* subcortical, spinal or peripheral regions) (Kojovic, Cordivari, & Bhatia, 2011).

Taken together, the *in vitro* and *in vivo* PTZ-associated data observed within this study recapitulates previously published *in vivo* PTZ-associated data observed in rats, NHPs and dogs (Bassett et al., 2014) where the rank order of PTZ-sensitivity (in terms of first convulsive episode) was dog (most sensitive) > rat > NHP. A similar rank order can be established within this investigation where PTZ-sensitivity within the *ex vivo* hippocampal seizure liability assay ranks as the Beagle dog (most sensitive) > rat (Sprague Dawley) > NHP (*i.e.* cynomolgus monkey) > Göttingen minipig. Beagle dogs are known to be overly sensitive to idiopathic epilepsy (Edmonds et al., 1979; Hoskins, 2000) with incidences up to 6% in inbred preclinical species (Easter et al., 2009). In addition, Beagle dogs require low PTZ concentrations (sub-40 mg/kg) to induce seizure (Bassett et al., 2014; Durmuller et al., 2007; van der Linde et al., 2011). Thus, it is unsurprising that Beagle dogs displayed such a high sensitivity to PTZ reaching statistical significance at lower PTZ concentrations (*e.g.* 3 mM) for both PS area and
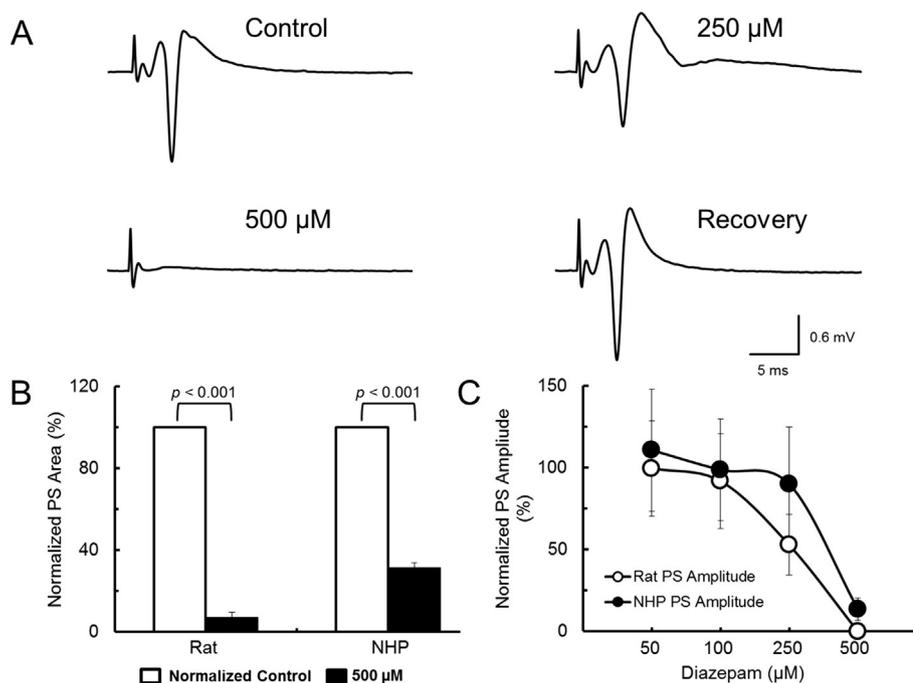
Fig. 5. Diazepam-induced changes on rat and NHP CA1 population spikes. A) Representative electrophysiological recordings of evoked PS in rat hippocampal tissue in the absence or presence of diazepam at a constant stimulation voltage. The recovery period was approximately 50 min. The scale is equivalent in all panels. B) Comparison of diazepam-induced changes to PS area in both rats (left, n = 3) and nonhuman primates (right, n = 3) at 500 μM diazepam. C) Comparison of the change in PS amplitude in the present of increasing diazepam concentrations within both rats (white circles) and nonhuman primates (black circles). All values have been normalized to the control condition. All error bars, ± S.E.M.

number. Given the genetic predisposition to idiopathic epilepsy in Beagle dogs, this species may possess limited translational relevance to assess seizure liabilities in humans. This high sensitivity of Beagle dogs carries major implications during safety margin estimation, a critical aspect of non-clinical seizure liability studies. However, it remains that seizure liability can, and is, evaluated in Beagle dogs routinely in non-clinical investigations particularly in cases when required by regulatory agencies following observations in canine toxicology studies. Rats and NHPs demonstrated similar sensitivities to PS area over the same active PTZ concentration range but rats routinely displayed greater PS

morphological changes such as PS number increases.

Further characterization of the rat and NHP hippocampal tissue generally demonstrated an increased sensitivity in rats to the pro-convulsant agents tested as they displayed larger increases in either PS area, PS number, or both. This elevated sensitivity to pro-convulsant agents may be associated with the greater overall number of GABAergic neurons found within NHP brain tissue compared to the rat (Yanez et al., 2005) which may influence the development and functionality of the hippocampus. A broader assessment covering additional pro- and anti-convulsant agents is required to get a fuller understanding of the
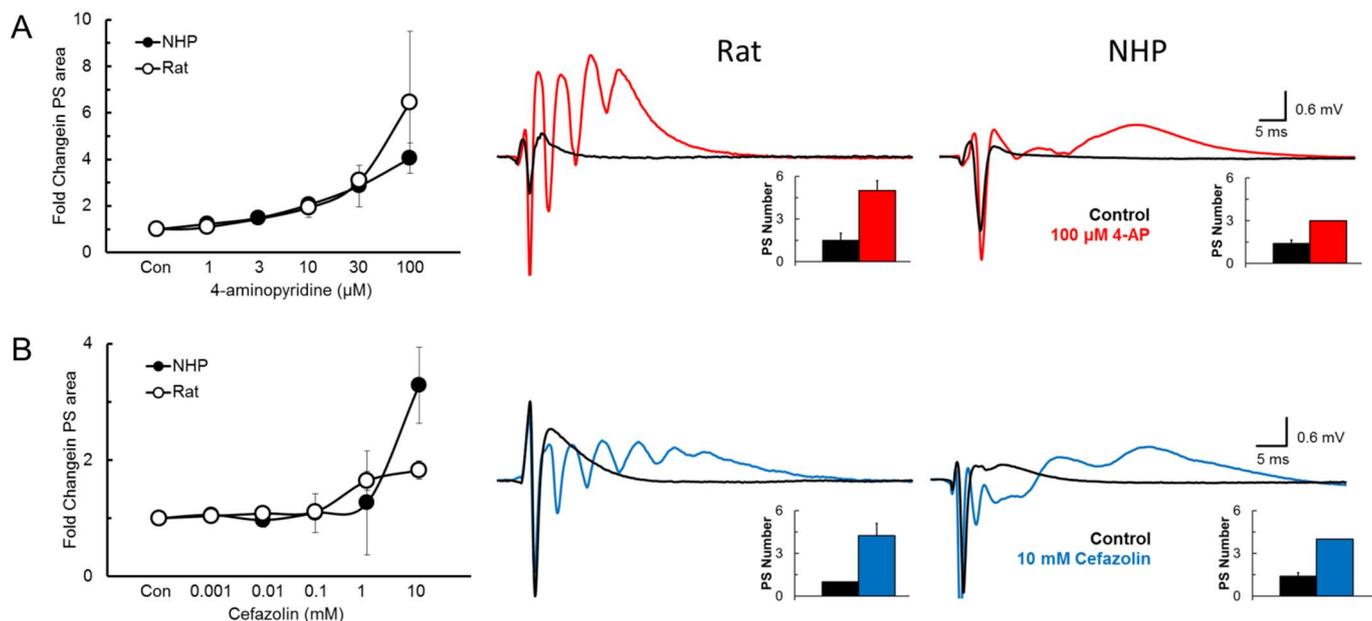

Fig. 6. Cefazolin and 4-AP-induced changes on rat and NHP CA1 population spikes. A) *Left*, Comparison of fold-change in PS area in increasing concentrations of 4-aminopyridine within both rats (white circles) and nonhuman primates (black circles). Representative electrophysiological recordings in the absence (black trace) and presence (red trace) of 100 μM 4-aminopyridane in both rats (middle) and nonhuman primates (right). The inset for each animal species highlights the change in PS number observed in both the control (black) and 100 μM 4-aminopyridane (red) conditions. B) *Left*, Comparison of fold-change in PS area in increasing concentrations of cefazolin within both rats (white circles) and nonhuman primates (black circles). Representative electrophysiological recordings in the absence (black trace) and presence (blue trace) of 10 mM cefazolin in both rats (middle) and nonhuman primates (right). The inset for each animal species highlights the change in PS number observed in both the control (black) and 10 mM cefazolin (blue) conditions. All error bars, ± S.E.M. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differences between these two species. However, this study highlights that both species are potential surrogates for the human condition within an *in vitro* seizure liability assay. For instance, cefazolin, a cephalosporin thought to modulate GABA-receptor activity (Akula, Dhir, & Kulkarni, 2007; Hori, Kurioka, Matsuda, & Shimada, 1985), has been shown to generate seizures markedly similar to that of PTZ (Hantson, Leonard, Maloteaux, & Mahieu, 1999) – an observation echoed within this investigation particularly within rat tissue. The predicated effects of 4-AP, a potassium channel blocker, and diazepam, a benzodiazepine, were also accurately identified within both rat and NHP tissue with the anti-convulsant effects of lidocaine (observed at low concentrations (DeToledo, 2000)) also demonstrated. Given the logistical implications concerning NHP research, rodent hippocampal tissue remains the most ethical approach to implanting an *in vitro* seizure liability assay routinely within early safety pharmacology programs. Nonetheless, NHP represent a much closer neuroanatomical and genetic surrogate for the human condition (Passingham, 2009; Preuss, 2000) and some safety programs may benefit from the use of cynomolgus monkeys when the *in vitro* rat hippocampal model is not appropriate to assess the seizurogenic activity of a given test article.

Safety pharmacology employs many testing strategies aimed at identifying seizure liability (Accardi et al., 2016; Authier et al., 2009, 2014). This is a particularly prominent concern within the safety pharmacology arena since seizures represent one of the most frequent causes of injury or death in human clinical trials (Bass et al., 2004). Despite this, there is no standard study or guideline for evaluating seizure liability preclinically with calls for more comprehensive CNS safety testing prior to clinical studies (Lindgren et al., 2008; Valentin & Hammond, 2008). Given the financial burdens of the drug discovery and safety testing process (DiMasi, Grabowski, & Hansen, 2016), frontloading *in vitro* brain slice models in selected programs may be considered to discriminate compounds with seizurogenic liabilities. Early implementation of the *in vitro* hippocampal/brain slice model within safety programs offers minimal use of compound and a broad test concentration range may be applied. However, it must be considered that *in vitro* brain slice models are also limited in their extrapolation to the *in vivo* condition since *ex vivo* brain slices may lack systemic influences that contribute to seizure onset (*e.g.* hormonal, inter-regional neuronal connections, temperature, metabolism, *etc.*) (Cole, Koh, & Zheng, 2002) or the site of action for the test article falls outside the brain region being investigated. While useful to identify the seizure risk, *in vitro* brain slices possess inherent limitations in their relevance to establish safety margins. *In vitro* brain slice result interpretation often relies on compound comparison (*i.e.* rank order) triggering the need for inclusion of reference drugs in the design. To this end, data obtained from *in vitro* hippocampal slice models is generally used as a complement, and not a substitute, for *in vivo* investigations aimed at influencing the design of subsequent non-clinical seizure liability studies. This is particularly important in early drug discovery programs with recognized seizure risk where an indication of seizure-like activity may lead to drug development termination.In other cases, *in vitro* hippocampal slice models may inform on the most relevant species to use in non-clinical drug safety evaluations. In conclusion, *in vitro* brain slices can be applied to compound and/or species comparison during seizure liability testing and may constitute an ethical surrogate to *in vivo* studies in early discovery.

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Conflicts of interest

None of the authors have any conflict of interest, other than their employment in a contract research organization. No information is presented in this paper that advocates for, or promotes, commercial products from the author's organization.

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