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The efficacy of sodium channel blockers to prevent PCP-induced cognitive dysfunction in the rat: Potential for novel treatments for schizophrenia

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Efficacy of Na channel blockers in NMDA antagonist models

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Non-standard Abbreviations: (CNS) central nervous system; DMSO (di-methylsulfoxide); GSK2 ((5R)-5-(4-{[(2-fluorophenyl)methyl]oxy}phenyl)-L-prolinamide); GSK3 ((2R,5R)-2-(4-{[(2-fluorophenyl)methyl]oxy}phenyl)-7-methyl-1,7-diazaspiro[4.4]nonan-6-one); hydroxypropyl-methylcellulose (HPMC); medial prefrontal cortex (mPFC); monoamine oxidase A or B (MAO-A or B); (NMDA) N-Methyl-D-aspartic acid; (phEEG) pharmaco-electroencephalography; (PCP) phencyclidine; relative cerebral blood volume (rCBV); volume of interest (VOI).

Abstract

Sodium channel inhibition is a well-precedented mechanism used to treat epilepsy and other hyperexcitability disorders. The established sodium channel blocker and broad-spectrum anticonvulsant, lamotrigine is also effective in the treatment of bipolar disorder, and has been evaluated in patients with schizophrenia. Double-blind placebo-controlled clinical trials found that the drug has potential to reduce cognitive symptoms of the disorder. However, due to compound-related side-effects and the need for dose-titration, a conclusive evaluation of the drug's efficacy in patients with schizophrenia has not been possible. GSK2 and GSK3 are two new, structurally diverse sodium channel blockers with potent anticonvulsant activity. In this series of studies in the rat, we compared the efficacy of the two new molecules to prevent a cognitive deficit induced by the NMDA receptor antagonist, PCP in the reversal-learning paradigm in the rat. We also explored the effects of the drugs to prevent brain activation and neurochemical effects of PCP. We found that both GSK2 and GSK3, like lamotrigine, were able to prevent the deficit in reversal learning produced by PCP, thus confirming their potential in the treatment of cognitive symptoms of schizophrenia. However, higher doses than those required for anticonvulsant efficacy of the drugs were needed for activity in the reversal learning model, suggesting a lower therapeutic window relative to mechanism-dependent central side effects for this indication.

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Introduction

Improved treatment for patients with schizophrenia requires either that we identify the genetic or molecular basis for the illness, or that we progress drugs with different mechanisms of action into patients to evaluate their efficacy in an empirical manner. This latter approach is facilitated by the use of models in which "psychosis" is induced by administration of NMDA receptor antagonists, such as ketamine or PCP in humans (Krystal, et al., 2002) or rodents (Large, 2007; Neill et al., 2010). These models are sensitive to atypical antipsychotic drugs. Lamotrigine, a voltage-gated sodium channel blocker that has broad spectrum anticonvulsant efficacy, and which is used in the treatment of bipolar disorder, has also been shown to reduce NMDA receptor antagonist-induced psychotic symptoms in both humans (Anand, et al., 2000; Deakin, et al., 2008) and rodents (Brody, et al., 2003; Gozzi, et al., 2008a; Hunt, et al., 2008; Idris, et al., 2005). These findings supported the proposition that the drug might be useful in the treatment of schizophrenia (Large, et al., 2005), a hypothesis that was also strongly supported by positive results from several small clinical trials of lamotrigine added on to atypical antipsychotic drugs in patients with treatment-resistant schizophrenia (Dursun, et al., 1999; Dursun and Deakin, 2001; Kremer, et al., 2004; Tiihonen, et al., 2003). Subsequently, two larger 12-week, double-blind placebo controlled trials with add-on lamotrigine failed to demonstrate efficacy on their primary endpoint (change in the Positive and Negative Symptom Scale), but did find evidence for improvement in cognitive symptoms (Goff, et al., 2007). Consistent cognitive benefit has been previously attributed to lamotrigine in patients with epilepsy (Aldenkamp and Baker, 2001). A large placebo response in the schizophrenia trials may have compromised their overall sensitivity, and the need for a 6-week dose-titration with lamotrigine in order to lower the risk of rash may also have reduced the likelihood of separating from placebo over the relatively short trial period. However, given the promise of efficacy of lamotrigine versus cognitive deficits in schizophrenia, which represent a major unmet need in the management of these patients, it has remained a key challenge to identify novel, structurally diverse compounds with a similar pharmacology that might be progressed into the clinic for this indication.

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As argued previously (Large, et al., 2005; Large, et al., 2009a; Xie and Hagan, 1998), the primary mechanism of action of lamotrigine is likely to be the use-dependent inhibition of voltage-gated sodium channels. Although other molecular interactions, such as inhibition of voltage-gated N-type (Cav2.2) calcium channels (Cunningham and Jones, 2000) and inhibition of monoamine-oxidase B (Southam, et al., 2005), cannot be ruled out as of possible therapeutic relevance. We have developed two novel use-dependent sodium channel blockers, GSK2 and GSK3. Like lamotrigine, the two compounds are effective anticonvulsant agents in animal models of seizure, but have notably higher affinities for the channels (Large, et al., 2009b), and are structurally distinct from the older drug and thus have a reduced risk of inducing rash. We therefore wished to examine their potential as agents for the treatment of cognitive deficits associated with schizophrenia.

The disruption of a cognitive task using NMDA receptor antagonists provides a useful model of the cognitive deficit observed in patients with schizophrenia. One such task, the “reversal learning” task, has been developed in rodents in order to assess rule learning and cognitive flexibility (Neill et al. 2010). PCP and other psychotomimetic drugs have been shown to selectively disrupt performance of the reversal phase of the task, where there is greater cognitive load. Importantly, this disruption can be prevented by atypical, but not typical, antipsychotic drugs (Neill et al., 2010). We therefore chose this model to compare the efficacy of lamotrigine with that of the new sodium channel blockers. The efficacy of lamotrigine in this model has been reported previously (Idris et al., 2005). In order to determine whether the new compounds have a similar profile to lamotrigine, and to increase our understanding of the mechanism behind the efficacy of the drugs versus PCP-induced cognitive disruption, we also investigated their ability to prevent the effects of PCP on frontal cortical monoamine levels and measures of brain activation. Finally, since central, mechanism-related side-effects often limit the potential to explore the efficacy of CNS-active drugs in patients, we also compared the liability of the three drugs to cause behavioural sedation or ataxia.

The study shows that, like lamotrigine, GSK2 and GSK3 could prevent the cognitive deficit induced by PCP in rodents. The new drugs also prevented brain-wide haemodynamic activation by PCP in a manner similar to lamotrigine, but unlike the older drug, had relatively little effect on the PCP-induced increase in frontal cortical monoamines. Analysis of the pharmacology of the new drugs suggests that their efficacy in the PCP models is unlikely to be due to a direct interaction with NMDA receptors, and is unaffected by the potent ability of GSK2 to inhibit monoamine oxidase B. These drugs show promise as new tools to explore the efficacy of sodium channel blockers in patients with schizophrenia.

Methods

Reversal Learning

Subjects

Eighty female hooded-Lister rats (Harlan, UK) were used as subjects for the present studies. Rats were housed in groups of 4-5 under standard laboratory conditions under a 12 hour light:dark cycle, lights on at 0700 hours. Testing was carried out in the light phase. Prior to operant training and testing, rats were gradually food deprived to approximately 90% of free-feeding body weight (225–250 g); reduced body weight was maintained by restricting the amount of food (standard laboratory chow, Special Diet Services, Essex UK) given to each rat per day (12 g/day). The availability of water was not restricted. Rats were aged 12 weeks at the start of training in the reversal learning paradigm and were 24 weeks old when the first drug treatment was given. Experiments were conducted in accordance with the Animals Scientific Procedures Act, UK, 1986, and approved by the University of Bradford ethical review process.

Drugs

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GSK2 was dissolved in 0.5% methylcellulose/2% DMSO. GSK2 was administered orally and was given 45 mins prior to testing and 15 mins prior to treatment with PCP. GSK3 was dissolved in saline and administered orally 90 mins prior to testing and 60 mins prior to treatment with PCP. Lamotrigine was dissolved in 0.5% methylcellulose and was administered via the intra-peritoneal route (i.p.) and given 90 mins prior to testing and 60 mins prior to treatment with PCP. PCP HCl (Sigma, UK) was dissolved in saline and administered i.p. 30 mins prior to testing. In all experiments, the appropriate vehicle treatment was used. All doses are quoted as base-equivalent weight.

Procedure

Training: Rats were trained and tested in the reversal learning paradigm as described previously (Idris et al., 2005). All rats were tested in one of eight operant chambers (constructed in-house) controlled by Med-PC software (Version 2.0 for DOS or Med-PC for Windows, Med Associates, Inc. Lafayette, Indiana). Following habituation to the operant chambers, rats were trained to respond for food on a fixed ratio 1 (FR1) schedule of reinforcement with both levers active. When responding stabilised, rats were trained to press either the left or right lever for food delivery according to a visual cue (LED on or off). These sessions took approx 30 mins and ended after 128 total responses (correct + incorrect). A criterion of 90% accuracy (≥115 correct responses per session) had to be achieved over at least 3 consecutive days before being trained on the opposite contingency. Approximately 6 weeks of daily training was required before introducing the reversal-learning task.

Testing: Each reversal-learning session began with a 5-min period (the initial phase) during which the reinforcement contingency matched that of the previous day's training session. At the conclusion of the initial phase, the house light was extinguished and a 2-min time-out began. After the time-out, the house light was illuminated, and the next phase of the task began (the reversal phase), during which the reinforcement contingency was reversed. That is, state of the visual cue (lit or unlit) associated with reinforcement was switched. The predictable nature of the time-out and the break in response requirement act as cues for the contingency change. These aspects of the task provide

the advantage of decreasing the time required for training. Several reversal-learning sessions were performed before initiating drug studies to ensure stable performance. A criterion of 75% accuracy had to be achieved over at least 3 consecutive days before drug testing. An additional week was required to perform reversal test sessions, before drug testing started.

Experimental design

Rats were tested on a cycle of 4 days (Idris et al., 2005). On day 1 each animal had a 30-min operant training session. The following day, animals received the appropriate drug(s) and undertook a reversal-learning session. On day 3 and day 4, each animal underwent a further operant training session and reversal task session, respectively, in order to ensure that responding was back to baseline after the drug treatment. The drug treatment given to each rat (and within each home cage) over the course of the seven experiments was randomised. Every effort was made to keep the number of drug and vehicle treatments equivalent among animals and to randomise the order of treatments. The second cohort took part only in the final drug experiment. This second cohort of animals was required in order to comply with the Animals Scientific Procedures Act (UK, 1986); i.e. there is a restriction in the number of drug treatments each animal can receive.

Data analysis

Data from this study are presented as the percent correct response (± s.e.m.), with values for the initial and reversal task presented for the different drug treatment groups. These data were used to determine whether there was a significant effect of drug(s) on response accuracy (e.g. that might reflect cognitive dysfunction); these data were Arc-Sin transformed prior to analysis. Statistical significance was assumed when $p < 0.05$ and was determined as follows: A one way ANOVA was performed in order to detect a main effect of drug(s) treatment on the initial and reversal tasks. Where a significant effect was detected, a post-hoc Dunnett's test was performed in order to compare treatment groups versus the appropriate control. Raw lever press data (Supplementary Tables) were used to determine whether there were significant effects of drug(s) on overall

responding that might reflect effects on motor co-ordination or motivation. These data were analysed in a similar manner to the percent correct response data.

Pharmacological Magnetic Resonance Imaging (phMRI)

Subjects

Functional imaging studies were performed on male Sprague-Dawley rats (250-350 g, Charles River, Como, Italy). Animals had free access to standard rat chow and tap water and were housed in groups of 5 in solid bottom cages with sawdust litter. Room temperature (20-22 °C), relative humidity (45-65%) and dark-light cycles (12 hours each, lights on at 06:00 hours) were automatically controlled. After arrival rats were allowed to acclimatize for at least five days. All phMRI studies were conducted in accordance with the Italian laws (DL 116, 1992 Ministero della Sanità, Roma). Animal research protocols were also reviewed and consented to by a local animal care committee, in accordance with the guidelines of the Principles of Laboratory Animal Care (NIH publication 86-23, revised 1985).

Animal preparation/monitoring and MRI acquisition in each phMRI study were similar to previous studies (Gozzi, et al., 2008b). Briefly, rats were anaesthetized with 3% halothane in a 30:70% O_2:N_2 gas mixture, tracheotomised and artificially ventilated with a mechanical respirator to ensure control of blood gas levels. The left femoral artery and vein were cannulated and animal paralysed with a 0.25 mg/kg i.v. bolus of D-tubocurarine followed by a continuous infusion of 0.25 mg/kg/h through the artery. A poly-ethylene cannula was inserted intraperitoneally or subcutaneously for drug pretreatment (see below). After surgery the rat was secured into a customized stereotactic holder (Bruker, Ettlingen, Germany) and the halothane level set to 0.8%. Arterial blood samples (0.5 ml) were taken immediately prior to and at the end of the functional MRI time series acquisition, and p_aCO_2 and p_aO_2 were measured using a blood gas analyser (Supplementary Table 3). All the subjects had p_aO_2 > 95 mmHg, corresponding to a hemoglobin saturation greater than 98%. No statistically significant difference ($p>0.05$) in mean pre- versus post-acquisition p_aCO_2 values for each pair of

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PCP-challenged groups was observed (one-way ANOVA, followed by Fisher's least significant difference (LSD) test for multiple comparisons). A magnetic resonance-compatible thermocouple probe was used to measure rectal temperature. The body temperature of all subjects was maintained within physiological range (37±0.8 ºC) throughout the experiment. Mean arterial blood pressure (MABP) was monitored continually through the femoral artery. At the end of the experiment, the animals were euthanised with an overdose of anaesthetic followed by cervical dislocation.

Relative Cerebral Blood Volume Measurements

MRI data were acquired using a Bruker Avance 4.7 Tesla system, a 72mm birdcage resonator for radiofrequency pulse transmit and a Bruker curved "Rat Brain" quadrature receive coil. The MR acquisition for each subject comprised T_2-weighted anatomical images using the RARE sequence (Hennig, et al., 1986) (TR_{eff} = 5000ms, TE_{eff} = 110 ms, RARE factor 8, FOV 40mm, 256x256 matrix, 16 contiguous 1mm slices) followed by a time series acquisition with the same spatial coverage and similar parameters (TR_{eff} = 2700 ms, TE_{eff} = 110 ms, RARE factor 32), but with a lower in-plane spatial resolution (128×128) giving a functional pixel volume of ~0.1mm^3. The use of T_2-weighted images for the time-series acquisition minimizes sensitivity both to large blood vessels and inhomogeneities of the static magnetic field (Boxerman, et al., 1995), and ensured that the geometry of the time-series images matched the anatomical reference images, facilitating subsequent image analysis. Two successive scans were averaged for a resulting time resolution of 40 secs. Following five reference images, 2.67ml/kg of the blood pool contrast agent Endorem (Guerbet, France) was injected so that subsequent signal changes would reflect alterations in rCBV (Schwarz, et al., 2003). Prior to the injection of drug pretreatment, an equilibration period of 15 mins was allowed. Experiments were performed with intraperitoneal (i.p.) or subcutaneous (s.c.) injection of drug (or vehicle) pretreatment followed by intravenous (i.v.) PCP challenge (or vehicle) 30 minutes later. All

compounds were injected at a rate of 1 ml/min. The MRI data were acquired over a period of at least 25 mins following the administration of the PCP challenge.

Experimental procedure and drugs

Phencyclidine hydrochloride (PCP; Sigma-Aldrich, Italy), dissolved in saline, was tested at a sub-anaesthetic dose (0.5 mg/kg i.v.) that produces robust and reproducible corti-colimbo-thalamic activation (Gozzi, et al., 2008b) and elicits substantial behavioral and metabolic (2-deoxyglucose) responses in freely-moving rats (Weissman, et al., 1987). In order to allow for a better randomisation and keep the study manageable, GSK2 and GSK3 were tested in separate studies that occurred at different times. Male SD rats were randomly assigned to one of the groups below.

a) GSK2 study

Group 1) s.c. preadministration of vehicle (HPMC 0.5 % in water, 4 ml/Kg) followed by i.v. challenge with PCP (0.5 mg/kg) 30 mins later (n=5).

Group 2) s.c. preadministration of GSK2 (30 mg/Kg) followed by i.v. challenge with PCP (0.5 mg/kg) 30 mins later (n=7).

Group 3) s.c. preadministration of GSK2 (100 mg/Kg) followed by i.v. challenge with PCP (0.5 mg/kg) 30 mins later (n=7).

Group 4) s.c. preadministration of vehicle (HPMC 0.5% in water) followed by an i.v. challenge with vehicle (saline, 1 ml/rat) 30 mins later (n=6). This group of rats served as reference (baseline) rCBV baseline for both experiments.

b) GSK3 study

Group 5) i.p. preadministration of vehicle (water, 2 ml/Kg) followed by an i.v. challenge with PCP (0.5 mg/kg) 30 mins later (n=9).

Group 6) i.p. preadministration of GSK3 (10 mg/kg) followed by i.v. challenge with PCP (0.5 mg/kg) 30 mins later (n=7).

Group 7) i.p. preadministration of GSK3 (30 mg/kg) followed by i.v. challenge with PCP (0.5 mg/kg) 30 mins later (n=6).

Group 8) i.p. preadministration of lamotrigine (lamotrigine isothionate, 10 mg/kg free base) followed by an i.v. challenge with PCP (0.5 mg/kg) 30 mins later (n=6)

Data analysis

The rCBV time series image data for each experiment were analyzed within the framework of the general linear model. Signal intensity changes in the time series were converted into fractional rCBV on a pixel-wise basis, using a constrained exponential model of the gradual elimination of contrast agent from the blood pool. Individual subjects in each study were spatially normalised by a 9-degree–of-freedom affine transformation mapping their T2-weighted anatomical images to a stereotaxic rat brain MRI template set (Schwarz, et al., 2006) and applying the resulting transformation matrix to the accompanying rCBV time series. rCBV time series for the PCP challenge were calculated covering 8 minute (12 timepoints) pre-injection baseline and 25 mins (38 timepoints) post-injection window, normalized to a common injection time point. rCBV time series were also calculated for the pretreatment covering 10 timepoints (≈ 6.6 minutes) pre-injection baseline and 30 timepoints (20 mins) post-injection window normalized to a common injection time point. Image based time series analysis was carried out using FMRI Expert Analysis Tool (Version 5.63), part of FMRIB's Software Library (www.fmrib.ox.ac.uk/fsl) with 0.8 mm spatial smoothing (≈ 2.5 x in-plane voxel dimension) and using a model function identified by wavelet cluster analysis across all PCP-treated animals, capturing the temporal profile of the signal change induced by PCP challenge in each group (Whitcher, et al., 2005; Schwarz, et al., 2007). As no substantial differences in the temporal profile of PCP-induced signal changes were observed across

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groups (see "Results" section), a common regressor was used for both studies (Supplementary Figure 3). Consistent with previous reports, PCP *per se* did not produce any significant sustained negative signal changes in any of the regions analysed (Gozzi, et al., 2008a). The design matrix also included the temporal derivative of this regressor and a linear ramp (both orthogonalised to the regressor of interest) with the aim to capture additional variance due to slight deviations in individual subjects or brain regions from the signal model time course as described in more detail elsewhere (Schwarz, et al., 2007). Higher-level group comparisons were carried out using FMRIB's Local Analysis of Mixed Effects. Z (Gaussianised T/F) statistic images were thresholded using clusters determined by $Z > 2.3$ and a corrected cluster significance threshold of $p = 0.01$ (Worsley, et al., 1992; Friston, et al., 1994).

VOI time courses for both the pretreatment and the PCP challenge were extracted from unsmoothed rCBV time series data using a 3D digital reconstruction of a rat brain atlas (Paxinos and Watson, 1998) co-registered with the MRI template (Schwarz, et al., 2006), using custom in-house software written in IDL (Research Systems Inc., Boulder, Colorado) For each VOI time course, the average rCBV over a 16 min time window covering the peak response to PCP (4-20 min post-injection) was used as a summary statistic of the relative change. Group rCBV response from VOIs was compared between different groups of treatment by a one-way ANOVA followed by a Dunnett's test *versus* group (1). Threshold for statistical significance was considered as p=0.05. Results are quoted and displayed as mean ± SEM unless otherwise indicated.

The rCBV time-profiles of the i.p. pretreatment *per se* did not show substantial signal changes compared to vehicle for all the compounds in 22 VOIs covering the main cortical and subcortical brain structures as previously described (Gozzi, et al., 2008a).

Administration of vehicle, GSK2, GSK3, lamotrigine, or PCP was accompanied by small and transient alterations of mean arterial blood pressure. In all cases, peak magnitude of the mean arterial blood pressure observed (Supplementary Table 6) was within the cerebral blood flow autoregulation range

measured under the same anaesthetic conditions used in the present study (Gozzi, et al., 2007). As shown by us and other groups, positive or negative pharmacologically evoked mean arterial blood pressure changes within the autoregulation range mentioned above do not result in significant central rCBV response when spin-echo MRI sequences are used (Gozzi, et al., 2007).

Neurochemistry

Subjects

Seventy male Sprague–Dawley rats (Charles River, Italy), originally weighing 225–300g, were housed at 21 ± 1 °C with 50% humidity on a 12-hour light:dark cycle in accordance with Italian law (art. 7, Legislative Decree no. 116, 27 January 1992) and the European Communities Council Directives (86/609/EEC).

Drugs

GSK2 was dissolved in methylcellulose (0.5% w/v) in sterile water. GSK3 was dissolved in methylcellulose (0.5% w/v) + DMSO (3% v/v) in sterile water. PCP (Sigma, Italy) was dissolved in ultra-high pure water. All injections were made s.c. in a volume of 1ml/kg body weight.

Experimental Procedure

Methods were similar to those previously described elsewhere (Quarta, et al., 2009). Subjects were anaesthetized and implanted in the mPFC (with respect to bregma: AP: +3.2 mm, ML: -0.5 mm and DV: -1.8 mm). Animals were then returned to their home cage and allowed at least seventy-two hours to recover from the surgery. Twenty-four hours after insertion of the microdialysis probe (MAB4, 4 mm active cuprophane membrane length, Agnthos, Stokholm, Sweden) a ringer solution consisting of (in mm) 125 NaCl, 2.5 KCl, 1.18 $MgCl_2 \cdot 6H_2O$, 1.3 $CaCl_2 \cdot 2H_2O$ and 2.0 Na_2HPO_4, (pH 7.4) was pumped through the probe at constant rate of 1.0 µl/min (Univentor 864 Syringe Pump, Agnthos, Stokholm, Sweden). After a 120-min wash period, three basal 20-min samples were

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automatically collected (Univentor 820 Microsampler, Agnthos, Stokholm, Sweden) over 60 mins. The animals were then injected with either vehicle, GSK2 (50, 100 mg/kg s.c.) or GSK3 (30 mg/kg s.c.) followed by PCP (1.5 mg/kg s.c.). Sample collection continued for three hours after PCP treatment. All samples were immediately frozen for subsequent analysis.

At the end of all experiments, rats were sacrificed and brains collected in 4% formaldehyde solution. Coronal sections were cut to verify probe location. Samples from rats with incorrect probe location were discarded from the final analysis. Monoamine content was measured by reverse high-performance liquid chromatography coupled to an electrochemical detector as described elsewhere. Raw data (expressed as pg/sample, not corrected for probe recovery) were converted to a percentage of baseline values (mean of the samples taken prior to drug or vehicle administration). Time-course data (monoamine levels) were statistically analyzed for each treatment using a two-way ANOVA with a between-subjects factor of drug dose and a repeated-measurements factor of time (Statistica Network 8.0 software, StatSoft, Tulsa, OK, USA). Differences between individual means were assessed with a planned comparison test. Statistical significance was set at $p \leq 0.05$ in all cases.

CNS Side Effect Assessment

Subjects

Male Sprague Dawley rats were obtained from Charles River (Como, Italy), weighing 250-300 g at test. Rats were housed in groups and left to acclimatize for a 1 week under standard laboratory conditions (23 ± 1°C; humidity 45-65%; light on 0600-1800 hours; food and water *ad libitum*). All tests were performed between 0900-1400 hours. All experiments were performed in accordance with European Community ethical regulations on the care of animals for scientific research (CEE Council 86/609 Italian D.L. 27/01/92 no. 116) and were fully compliant with GSK ethical standards.

Drugs

GSK2 was suspended in HPMC (0.5% w/v, Colorcon Dow Chemical Company, UK) and Tween80 (0.1%, v/v; Sigma, Italy). GSK3 was dissolved in 1% or 3% DMSO (v/v) and then suspended in HPMC (0.5% w/v) to the final volume. Lamotrigine (supplied by GSK Medicinal Chemistry) was dissolved in DMSO (1% v/v) and HPMC (0.5% w/v). All doses and concentrations are expressed in terms of the parent compound.

Procedures

Rat motor behaviour was assessed during the light period using automated activity chambers (Omnitech Instruments, model RXYZCM-8, OH, USA). Each monitor consisted of a 41 cm × 41 cm × 30.5 cm Plexiglas arena covered by a Plexiglas lid with air holes. Each arena was surrounded by two sets of infrared beams for activity measurement with each set consisting to 16 beams located at 2.54 cm intervals around the four sides of the arena. These were located 4.5 cm above the floor for measurement of "horizontal activity" and 15 cm above the floor of the arena for measurement of "vertical activity". A Digiscan Analyzer (Omnitech, Model DCM-4, OH, USA) detected beams broken by the animal and thus determined the location of the rodent within the arena. The main variables recorded by the automated system were horizontal and vertical activity and were expressed as the total number of beam interruptions that occurred in the horizontal/vertical sensors. These variables were measured in 5-min bins across the 30-min session.

Studies were performed according to a blind, randomized design. GSK2 was administered p.o. (5 mL/kg body weight) to rats at the doses of 60 (n=8), 100 (n=8) and 300 mg/kg (n=8), 30 mins before the test session. GSK3 was administered s.c. (2 mL/kg body weight) to rats at the doses of 30, 60 and 100 mg/kg (n=9 per group), 30 mins before the test session. Lamotrigine was given p.o. to rats at 3, 10 and 30 mg/kg (n=9 per group), 60 mins before test session. Control rats (n=8-9) were given an equal volume of the respective vehicle by the appropriate route at the appropriate time.

Determination of the plasma concentration of each drug was carried out on a subset (n=6 per group) of the animals used in the behavioral test. Blood samples were taken at 60 mins (GSK2 or GSK3) or 90 mins (lamotrigine) after drug treatment (at the end of the test period). Blood samples were analyzed for drug concentration using a method based on protein precipitation followed by liquid chromatography/mass spectrometry; plasma concentrations were calculated based on the plasma protein binding for each drug determined from equilibrium dialysis studies (described in Large et al., 2009b), and corrected for the blood:plasma ratio as appropriate.

Data Analysis

Statistical analyses were conducted using GB-Stat v7 (Dynamic Microsoft Systems, Inc., MD, U.S.A.) Behavioral data were analyzed by one-way ANOVA, followed by a one tailed Dunnett`s *post hoc* test. Results were expressed as the observed mean ± S.E.M.. Differences between groups were considered to be statistically significant if the *p* value was less than 0.05.

NMDA receptor inhibition

Whole-cell patch clamp recordings were made from rat dissociated cortical neurones, (E18, maintained in culture for 7-8 days prior to electrophysiological recordings) voltage-clamped at -60 mV. NMDA receptor-mediated responses were activated by co-applying 50 μM NMDA and 10 μM glycine, every 50 s. The extracellular recording solution comprised (in mM): NaCl 130, KCl 5, CaCl2 2, Glucose 30, HEPES 25, pH7.3 (adjusted with NaOH) and the intracellular solution comprised: CsCl 140, MgCl2 4, HEPES 10, EGTA 10, pH7.3 (adjusted with CsOH). Drugs were prepared as 100 mM stocks in DMSO and both pre-applied in extracellular solution and co-applied with NMDA and glycine.

Monoamine oxidase inhibition

Brain Homogenate Preparations

Adult male CD rats (250-300 g, Charles River, Italy) were used to prepare forebrain homogenate as previously reported (Southam et al., 2005). Animals were killed by decapitation and forebrains were quickly removed and placed in ice-cold 154 mM sodium phosphate buffer (pH 7.8). Tissues were homogenised using Ultra-Turrax T8 homogenizer (IKA™-WERKE) and then centrifuged 2000x*g* for 10 min at 4°C. The supernatant was rapidly frozen in dry-ice and kept at -80°C until use. Protein concentration was measured using a Bio-Rad Protein Assay Kit (BIO-RAD, Italy).

In vivo Rat Treatment for Ex Vivo Studies

Adult male CD rats (250-300 g, Charles River, Italy, 4-5 per group) were used. GSK2 was administered (2ml/kg body weight) at 0.3, 3, and 10 mg/kg s.c. in 0.5% w/v methylcellulose in water in a first experiment, and at 3, 10, and 30 mg/kg s.c. in 1% v/v DMSO/0.5% w/v methylcellulose in water in a second experiment. In both experiments, control rats were administered with vehicle. Thirty minutes or 24 hours after treatment, rats were killed by decapitation, the forebrains were removed and homogenates prepared as above.

Drugs and Materials

Clorgyline, L-deprenyl were obtained from Sigma (Italy). [^{14}C]-benzylamine (specific activity 2.00 GBq/mmol) was from Amersham (Italy) and [^{3}H]-5-Hydroxytriptamine creatine sulphate (specific activity 1.11 TBq/mmol or 0.7511 TBq/mmol) was from PerkinElmer, Italy. The Amplex™ Red Monoamine Oxidase Assay Kit and Amplex™ Red substrate were from Invitrogen. Recombinant human MAO-A and MAO-B were obtained from Sigma-Aldrich (Spain).

MAO-A and MAO-B Assays

MAO-A and MAO-B activities using human recombinant enzymes were assayed by measuring the co-product, hydrogen peroxide (H_2O_2), that is produced during the deamination of substrate. In the presence of excess N-acetyl-3,7-dihydroxyphenoxazine (Amplex™ Red, Molecular Probes) and horseradish peroxidase, each molecule of peroxide produced converts one molecule of Amplex™ Red

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into the stable fluorescent product resorufin. Assays were performed in duplicate or triplicate in a final volume of 10 µl using black 384-well plates containing 0.23 IU/ml of human MAO-B or 0.28 IU/ml of human MAO-A recombinant enzymes and incubated for 1 h at room temperature in the dark. For the MAO-B assay the substrate was 400 µM benzylamine and for MAO-A the substrate was 200 µM p-tyramine. 50 µM Amplex™ Red and 1 IU/ml of commercial Horseradish Peroxidase (HRP) in 50 mM potassium phosphate buffer at pH 7.4 were added to the plates with the appropriate substrate and test compound. Reaction was started by the addition of the enzymes and fluorescence was measured after 60 min in the dark using a fluorescent plate reader with the excitation and emission filters at 555 nm and 590 nm respectively. MAO-A and MAO-B activities were expressed as percentage of inhibition (% inhibition).

Native MAO assays were performed in triplicate in a final volume of 100 µl phosphate buffer saline (pH 7.4; GIBCO) containing 0.05 mg protein (rat forebrain homogenates) and incubated at 37°C for 10 minutes as previously described (Southam et al., 2005). For the MAO-A assay the substrate was 5 µM [3H]-5-HT and for MAO-B the substrate was 13.5 µM [14C]-benzylamine. MAO-A or B isoform activity was pharmacologically isolated by inclusion in the reaction mix of 1 µM L-deprenyl or 1 µM clorgyline, respectively. Assays were terminated by the addition of 2 M HCl and deaminated reaction products extracted by mixing with 600 µl 1:1 v/v ethyl acetate/toluene. Samples were centrifuged at 8000xg for 2 min at room temperature; in duplicate, 200 µl of solvent phase was added to 3.5 ml scintillation fluid and radioactivity was determined by β-Counter scintillation counting. Non-specific activity was determined by adding HCl before the forebrain homogenate. The amount of MAO-A and MAO-B activity was expressed as nmolar/mg protein/min for substrate dependence *in vitro* determinations and *ex vivo* experiments, and as percentage of control activity (% control activity) for *in vitro* concentration inhibition determinations.

Data Analysis

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V_{max} and K_m parameters were obtained from *in vitro* substrate concentration dependence curves using a nonlinear regression fit to the Michaelis-Menten equation (GraphPad Prism™ version 4). pIC_{50} values were calculated from *in vitro* concentration inhibition curves using nonlinear regression fit to a sigmoidal dose-response curve (GraphPad Prism™ version 4). pK_i values were calculated from IC_{50} values using the Cheng-Prusoff equation and calculated K_m values from *in vitro* substrate concentration dependence curves. Statistical analysis was conducted using GraphPad Prism™ (version 4). Data derived from *ex vivo* studies on MAO activity were analysed by One-way ANOVA, followed by a Dunnett's *post-hoc* test. Differences between groups were considered to be statistically significant if the *p* value was less than 0.05.

Results

Reversal Learning

Experiment 1: A one way ANOVA of the experiment indicated that there was no significant effect of PCP or GSK2 treatment on correct responding in the initial phase [F (5, 97) = 0.41, NS]. However, a one way ANOVA showed that there was a significant effect of drug treatment on correct responding in the reversal phase [F (5, 97) =9.6, $p < 0.001$], such that animals showed a significant difference in correct responses between drug treatment groups (Figure 1a). Post-hoc analysis showed that PCP alone significantly reduced correct responding in the reversal phase ($p < 0.001$) when compared with vehicle. GSK2 (20 to 80 mg/kg p.o.) dose-dependently attenuated the deficit in reversal learning induced by PCP. This reduction was significantly attenuated by GSK2 at 60 mg/kg and at 80 mg/kg ($p < 0.05$, $p < 0.001$, respectively) when compared with PCP alone. Neither PCP alone nor GSK2 at 20-80 mg/kg plus PCP had a significant effect on the total number of lever presses in either the initial or reversal phases of the task [F (5, 97) = 0.85, NS], [F (5, 97) = 0.94, NS], respectively (Supplementary

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Table 2). Furthermore, GSK2 had no effect alone on either the initial or reversal phase of the task or on lever pressing (Supplementary Figure 1, Supplementary Table 1).

Experiment 2: A one way ANOVA of the experiment indicated that there was no significant effect of PCP or GSK3 treatment on correct responding in the initial phase [F (4, 49) = 0.98, NS]. However, a one way ANOVA revealed a significant effect of drug treatment on correct responding in the reversal phase [F (4, 49) =4.0, $p < 0.01$], such that animals showed a significant difference in correct responses between drug treatment groups (Figure 1b). Post-hoc analysis showed that PCP alone significantly reduced correct responding in the reversal phase ($p < 0.01$) when compared with vehicle. GSK3 (10-60mg/kg p.o.) dose-dependently reduced the deficit produced by PCP with a significant reduction in the deficit at 30 mg/kg and 60 mg/kg ($p < 0.05$, $p < 0.01$, respectively). Neither PCP alone nor GSK3 with PCP significantly affected lever pressing in the initial or reversal phases of the task [F (4, 49) = 1.8, NS], [F (4, 49) = 1.9, NS] (Supplementary Table 4). Furthermore, GSK3 had no effect alone on either the initial or reversal phases of the task or on lever pressing (Supplementary Figure 2, Supplementary Table 3).

Experiment 3: In a final experiment, conducted with the same groups of rats, a single dose of lamotrigine (25 mg/kg p.o.) was tested. A one way ANOVA of the experiment indicated that there was no significant effect of PCP or lamotrigine treatment on correct responding in the initial phase [F (3, 39) = 0.38, NS]. However, a one way ANOVA revealed a significant effect of drug treatment on correct responding in the reversal phase [F (3, 39) =3.9, $p < 0.05$]. As expected, lamotrigine significantly prevented the deficit induced by PCP in the reversal phase of the task ($p < 0.05$) (Figure 1c). Lamotrigine alone or with PCP did not significantly affect lever pressing (Supplementary Table 5). Furthermore, lamotrigine had no effect alone on either the initial or reversal phases of the task or on lever pressing.

Functional Neuroimaging

PCP administration (groups 1 and 5) produced a robust and sustained rCBV response in cortical (prefrontal, orbitofrontal, cingulate/retrosplenial cortices) and subcortical (frontal regions of the caudate putamen, nucleus accumbens, ventromedial and dorsolateral thalamus, ventral hippocampus) structures (Figure 2). The overall temporal profile of PCP-induced activation was comparable in all the activated regions with a sustained response that lasted throughout the 25 minute time-window examined (Supplementary Figures 3-5). Despite differences in the peak magnitude of PCP response across studies, the spatial distribution and relative amplitudes of the regional response to PCP were consistent and conserved across the different control groups (groups 1 and 5; Figure 3).

Pre-treatment with GSK2 (30 or 100 mg/kg s.c.) produced a dose-dependent reduction in the rCBV response to PCP with near-complete response inhibition at the highest dose (Figures 2a). These effects were also evident on rCBV timecourse plots (Supplementary Figure 3). No areas of increased response to PCP were observed in any of the GSK2-pretreated groups.

Pre-treatment with GSK3 (10 or 30 mg/kg i.p.) produced a dose-dependent reduction in the rCBV response to PCP with near-complete response inhibition at the highest dose (Figures 2b, Supplementary Figure 4). These effects were also evidence from the VOI-based analysis (Figure 3b). Pre-treatment with lamotrigine (10 mg/kg i.p.) also produced a profound inhibition of PCP-induced activation (Figure 2b, Supplementary Figure 5) that was evident in both the activation maps and VOI-based analysis (Figure 3b).

Pre-administration of GSK2, GSK3, lamotrigine or vehicle *per se* did not produce substantial alterations of basal rCBV with respect to vehicle in any of the regions examined (Supplementary Figures 6-8). This finding rules out a “ceiling” or “floor” rCBV effects in the response inhibition produced by the pretreatments.

Neurochemistry

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GSK2 (50 – 100 mg/kg s.c.) had no effect on extracellular levels of either NA, DA or 5-HT in the mPFC in the 60 minutes period prior to s.c. administration of 1.5 mg/kg PCP ($F_{(3, 17)} = 2.38$; NS, $F_{(3, 22)} = 2.91$; NS, $F_{(3, 20)} = 0.21$; NS, respectively). Systemic administration of PCP induced an increase in the extracellular levels of all monoamines, although this was significant over the full time-course only for NA in this experiment (Figure 4a). Consistent with this, a two-way ANOVA applied to the post-treatment NA values showed a significant effect of treatment ($F_{(3, 17)} = 13.83$ $p < 0.01$), as well as time ($F_{(8,136)} = 6.48$ $p < 0.01$). A two-way ANOVA applied to the post-treatment DA values showed a significant effect of time ($F_{(8,176)} = 16.22$ $p < 0.01$), but not treatment, although there was a time by treatment interaction ($F_{(24, 176)} = 1.62$ $p<0.05$), consistent with an increase in DA levels due to PCP that was relatively transient, as can be appreciated from Figure 4b. In contrast, a two-way ANOVA applied to the post-treatment 5-HT values showed only a significant effect of time ($F_{(8,160)} = 4.68$ $p < 0.01$), although the increase at specific timepoints was close to significance ($p = 0.09$). Comparisons between the vehicle+vehicle groups and drug-treatment groups were conducted using a multiple comparison test at each time point, with significant differences indicated by the stars in Figure 4. A similar comparison between the vehicle+PCP groups and GSK2-treated groups found no significant differences, suggesting no effect of GSK2 on PCP-induced increases in monoamine levels.

GSK3 (30 mg/kg s.c.) had no effect on extracellular levels of either NA, DA or 5-HT in the mPFC in the 40 minutes period prior to s.c. administration of 1.5 mg/kg PCP ($F_{(2, 18)} = 1.44$; NS, $F_{(2, 21)} = 0.14$; NS, $F_{(2, 19)} = 0.46$; NS, respectively). In this experiment, systemic administration of PCP induced a significant increase in the extracellular levels of all monoamines in the rat mPFC (Figure 5). A two-way ANOVA applied to the post-treatment NA values showed a significant effect of treatment ($F_{(2, 18)} = 8.71$ $p < 0.01$) and time ($F_{(8,144)} = 7.35$ $p < 0.01$), as well as a time by treatment interaction ($F_{(16,144)} = 1.92$ $p < 0.05$). Two-way ANOVA applied to the post-treatment DA values showed a significant effect of treatment ($F_{(2, 21)} = 4.74$ $p<0.05$) and time ($F_{(8,168)} = 5.47$ $p < 0.01$). Two-way ANOVA applied to the post-treatment 5-HT values also showed a significant effect of treatment ($F_{(2, 19)} = 3.59$ $p<0.05$) and time ($F_{(8,152)} = 2.60$ $p<0.01$), as well as a time by treatment interaction ($F_{(16,152)} = 2.60$

p<0.01). The significance of comparisons between the vehicle+vehicle groups and drug-treated groups at each time point are indicated in Figure 5. Comparisons between the vehicle+PCP group and the GSK3-treated group found no differences.

Motor Behaviour

One way ANOVA revealed that there was a statistically significant effect of GSK2 treatment on vertical activity [F (3, 28) = 5.5, $p < 0.01$], with the two highest doses of the drug (100 and 300 mg/kg p.o.) significantly reducing activity compared to vehicle-treated rats ($p < 0.05$ & $p < 0.01$, respectively; Figure 6a). A similar trend toward a reduction was also observed in horizontal activity following treatment [F (3, 28) = 3.4, $p < 0.05$]; however, the reduction was statistically significant compared to vehicle-treated rats only at the highest dose ($p < 0.05$; Figure 6a). At 60 mg/kg, the highest dose that did not significantly affect either horizontal or vertical activity, the free plasma concentration of GSK2 was 786 ng/mL (Total blood 7863 ng/mL; the blood:plasma ratio was 0.6 and plasma protein binding was 94%) (Table1).

GSK3 significantly reduced vertical activity [F (3, 32) = 5.75, $p < 0.01$] at all doses tested (30, 60, and 100 mg/kg s.c.; $p < 0.05$, $p < 0.01$ & $p < 0.01$ vs. veh, respectively; Figure 6b). The effect of treatment on horizontal activity was marginally significant [F (3, 32) = 2.3, $p < 0.10$]: post-hoc analysis showed that animals treated with the highest dose of GSK3 (100 mg/kg s.c.) had a lower horizontal activity than vehicle-treated rats ($p < 0.05$; Figure 6b). The mean free plasma concentration of GSK3 at the lowest dose tested, 30 mg/kg s.c., was 325 ng/mL (Total blood 6509 ng/mL; the blood:plasma ratio was 0.62 and plasma protein binding was 96.9%) (Table1).

Lamotrigine significantly reduced vertical activity [F (3, 32) = 3.04, $p < 0.05$] at all doses tested (p <0.05 vs. veh), but had no effect on horizontal activity at any dose [F (3, 32) = 1.26 p= 0.31] (Figure 6c). The dose of 3 mg/kg was associated with a plasma concentration of 675 ng/mL (Total blood 1377 ng/mL; the blood: plasma ratio was 1 and plasma protein binding was 51%) Table1).

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NMDA receptor antagonism

The application of GSK2 (100 μM) to dissociated rat cortical neurons inhibited whole-cell currents mediated by NMDA receptor activation. Mean current amplitude was reduced by 29 ± 4% (n=5, Supplementary Figure 9a). Similarly, the application of GSK3 (100 μM) inhibited whole-cell NMDA receptor-mediated currents by 20 ± 4% (n=4, Supplementary Figure 9b).

Monoamine oxidase inhibition

Initial studies with human recombinant MAO-A and B showed that GSK2 was a potent inhibitor of human MAO-B (pIC50 = 7.96±0.12, n= 4), but did not inhibit human MAO-A (pIC50 = 4.39±0.03, n= 3). In contrast, GSK3 had no activity at either enzyme (pIC50 <4, n=2 in each case). No further studies were conducted with GSK3. The ability of GSK2 to inhibit MAO-A and MAO-B activity in rat forebrain homogenate was assayed using [^{3}H]5-HT (in the presence of 1 μM selegeline) and [^{14}C]benzylamine (in the presence of 1 μM clorgyline), respectively.

Substrate dependence of MAO activity was first determined, giving Km values of 75.9 ± 4.71 and 93.2 ± 8.3 μM at MAO-A and B, respectively (n=3), and velocity values (V_{max}) of 1.106 ± 0.05 and 0.831 ± 0.07 nmol/mg/min, respectively (n=3). GSK2 inhibited rat forebrain MAO-B with a pKi of 7.20 ± 0.09 (n=3), similar to the standard inhibitor, L-deprenyl (pKi 7.15 ± 0.08, n=3). In contrast, GSK2 displayed low affinity for MAO-A enzyme in rat brain homogenate (pKi <5, n=3). Whereas, the selective inhibitor clorgyline showed the expected affinity (pKi 7.6, n=2).

Given the activity of GSK2 versus MAO-B, further studies evaluated the potency of the drug to inhibit the enzyme *in vivo*. GSK2 significantly inhibited rat forebrain MAO-B activity, *ex vivo*, at all doses tested (p < 0.05 using repeated measured ANOVA analysis with Post-Hoc Dunnetts testing) 30 minutes after treatment (T_{max}) (Figure 7). The highest dose of GSK2 (30 mg/kg s.c.) resulted in MAO-B enzyme inhibition of 65% (0.039±0.004 vs. 0.013±0.002 nmol/mg/min, vehicle vs. 30 mg/Kg treated animals). Twenty four hours after dosing with GSK2 (3mg/kg s.c.), MAO-B enzyme activity in

rat forebrain was comparable to vehicle treated animals (0.045±0.009 vs. 0.048±0.004 nmol/mg/min, vehicle vs. 3 mg/Kg treated animals). GSK2 did not inhibit MAO-A activity in rat brain homogenate, *ex vivo*, at any dose tested (data not shown).

Discussion

We investigated the ability of two novel use-dependent sodium channel blockers, GSK2 and GSK3 to prevent a cognitive deficit induced by the NMDA receptor antagonist, PCP in rodents. Results from the reversal learning model showed that, like lamotrigine (Idris et al., 2005), both GSK2 and GSK3 attenuated the disruption of the cognitive task by PCP. We have shown previously that atypical antipsychotic agents could attenuate the deficit produced by PCP; whereas, classical antipsychotics did not (Idris et al., 2005). These findings suggest that GSK2 and GSK3, like lamotrigine, have similarities to atypical antipsychotic agents in this regard. Low doses of PCP and ketamine are thought to produce psychotomimetic effects through selective blockade of NMDA receptors located on inhibitory interneurons within the cortex (reviewed in Large, 2007). Thus, reduced local circuit inhibition may lead to an increase in excitability and a decrease in firing precision of principal glutamatergic neurons. In support of this, ketamine has been shown to increase glutamate release in the cortex (Moghaddam et al, 1997) and alter the firing pattern of cortical projection neurons (Jackson et al, 2004). It has been hypothesized that the efficacy of lamotrigine in models of aberrant behavior induced by PCP or ketamine is most likely due to sodium channel inhibition, which can normalize the activity of glutamatergic neurons in the cortex (Large, et al., 2005). The results of the present study with GSK2 and GSK3, two structurally unrelated sodium channel blockers, are consistent with this hypothesis.

Lamotrigine was effective in the reversal learning model at plasma concentrations that were 3.7-fold higher than those required for anticonvulsant efficacy (Table 1 and Large et al., 2009b). Similarly,

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GSK3 required plasma concentrations approximately 4-fold higher than those producing a significant anticonvulsant effect; GSK2, however, required more than 14-fold higher plasma concentrations for efficacy in the reversal learning model compared to the electroshock model (Table 1 and Large et al., 2009b). The active plasma concentrations quoted for the reversal learning model (Table 1) are based on estimates from separate pharmacokinetic studies in rat using the appropriate route of administration for each drug. Given the variability typical of such measures across studies, it will be necessary to confirm these concentrations in a dedicated pharmacokinetic analysis in the reversal learning model. However, given the consistent increase in required plasma concentration for each of the three drugs, it seems likely that prevention of the PCP-induced cognitive deficit does require higher levels of the sodium channel blocking drugs compared to their anticonvulsant potency. Previously we showed that the anticonvulsant potency of the three drugs in the electroshock seizure model was consistent with their in vitro sodium channel affinities (Table 1; Large et al., 2009b), taking into account their respective plasma protein and brain tissue binding. We suggest that a similar relationship might hold for the reversal learning model, albeit at higher drug levels, for lamotrigine and GSK3. However, in the case of GSK2 the much greater fold-increase in plasma concentration required for efficacy in the reversal learning model compared to the drugs anticonvulsant potency suggests that other factors may be involved.

In order to shed further light on these possible differences, we profiled GSK2, GSK3, and lamotrigine in a series of mechanistic studies. For practical reasons it was not always possible to use identical routes of drug administration for the different in vivo studies; however, we used prior pharmacokinetic data to select doses that would achieve comparable exposures or else we used at least 2 doses in each model to ensure that we covered a sufficient range of exposure to draw conclusions regarding efficacy. We used phMRI to profile the brain-wide haemodynamic response to an acute challenge with PCP in order to look for regional effects that might be associated with the reversal learning results. The pattern of activation produced by PCP was consistent with that observed in our previous studies (Gozzi et al., 2008a), and similar to the pattern of [^{14}C]-2-

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deoxyglucose uptake induced by NMDA receptor antagonists (Duncan, et al., 1999). A significant increase in haemodynamic activity in frontal cortical areas is consistent with the impact of PCP on cognitive function in the reversal learning task. These experiments confirm our previous finding that lamotrigine can fully inhibit the central haemodynamic response to a psychotomimetic dose of PCP (Gozzi et al., 2008a). GSK2 and GSK3 were also able to fully prevent the functional activation elicited by PCP, with a similar brain-wide pattern of inhibition. This brain-wide pattern of inhibition contrasts with that of the atypical antipsychotics tested in this model: Clozapine, for example, produced a greater inhibition of thalamic activation, with less effect on cortical PCP-induced activation (Gozzi et al., 2008a). Brain-wide inhibition of the PCP-induced response suggests that the point of intervention of the sodium channel blockers is closer to the site of action of PCP compared to the antipsychotic drugs. However, this also raises the concern that the drugs might simply interfere with PCP inhibition of NMDA receptors. We have previously shown that neither GSK2 nor GSK3 displace binding of a competitive NMDA receptor antagonist, CGP39653 to adult rat cortical homogenates (<10% inhibition of specific binding at 10μM of each compound). In the present study, we used an electrophysiological assay to show that neither GSK2 nor GSK3 inhibit NMDA receptor-mediated currents in cultured cortical neurons. A caveat to these functional studies is that the NMDA receptor subunit composition of the embryonic cortical neurons differs from the adult, thus we cannot rule out the possibility that GSK2 and 3 might have a greater effect on NR2A subunits that are predominant in the adult, compared to the NR2B subunits that are predominant in our test system. However, given that only a small reduction (<30%) in NMDA currents was observed in the cortical neuron preparation in the presence of concentrations of GSK2 or GSK3 nearly 100-fold higher than those required for efficacy in the behavioral model, we conclude that direct interaction with NMDA receptors is unlikely to account for their in vivo efficacy.

In contrast to the similarities in efficacy of the two new drugs compared to lamotrigine in the reversal learning and phMRI studies, there were differences when comparing the ability of the drugs to prevent PCP-induced neurochemical changes in frontal cortex. Lamotrigine significantly reduced

PCP-induced overflow of DA and 5-HT, with a strong trend to reduce NA levels, but had no effect on locomotor hyperactivity (Quarta and Large, 2010). Neither GSK2 nor GSK3 significantly reduced the PCP-induced increase in any of the monoamines (comparing vehicle+PCP to GSK+PCP groups), although with GSK3 there was perhaps a trend for a reduction in NA and DA. The absence of significant effects of the new compounds on PCP-induced monoamine overflow compared to lamotrigine could be due to variability in the response to the psychotomimetic. For example, the increase in serotonin in the first experiment (Figure 4c) was less robust compared to the second (Figure 5c). However, the monoamine increases observed in both of the present experiments were numerically similar to those observed in the previous lamotrigine study (Quarta and Large, 2010). Insufficient dose of GSK2 or GSK3 relative to lamotrigine also seems unlikely, since the subcutaneous doses chosen would achieve higher exposures than the oral doses that were effective in the reversal learning model. Thus these results suggest that, whereas disruption of reversal learning and regional metabolic activation induced by PCP are robustly prevented by the new sodium channel blockers, PCP-induced monoamine overflow is at best only weakly blocked. A dissociation between locomotor hyperactivity and increased cortical monoamine levels induced by NMDA receptor antagonists has been shown previously (Adams and Moghaddam, 1998). Our studies now suggest an extension of this dissociation to include the cognitive disruption caused by NMDA receptor antagonists. The absence of effect of GSK2 and GSK3 also suggests that PCP-induced monoamine overflow is less sensitive to sodium channel blockade than the other consequences of NMDA receptor antagonism. We can speculate that PCP-induced monoamine overflow is driven by effects of the psychotomimetic directly within brainstem monoamine nuclei, and not as a consequence of increased glutamate release from cortical afferents to these areas (see Figure 2 in Large, 2007). However, given the contrasting result from our earlier study which found that lamotrigine could significantly reduce PCP-induced increases in at least DA and 5-HT (Quarta and Large, 2010), further evaluation of this aspect of NMDA receptor antagonist models is required.

Given the higher fold-increase in concentration of GSK2 required for efficacy in the reversal learning model compared to GSK3 and lamotrigine, we wondered whether interaction of GSK2 with monoamine oxidase B might be a factor, since lamotrigine (Southam et al., 2005) and GSK3 (data not shown) have little or no interaction with this enzyme. GSK2, at doses below those that were effective in the reversal learning model, significantly inhibited MAO-B activity *in vivo*; therefore, we can assume that significant inhibition of the enzyme will have occurred at the doses used in the reversal learning model, phMRI and neurochemical studies. The drug alone did not affect monoamine levels in the neurochemistry study; however, this would be consistent with previous studies with other MAO-B inhibitors (e.g. Lamensdorf, et al., 1996). The physiological role of MAO-B in the CNS is less well studied than MAO-A, in part due to the absence of very selective inhibitors, although MAO-B inhibitors, such as rasagiline, have been progressed for adjunctive treatment of Parkinson's disease (Hauser, 2009), suggesting some effect on dopamine disposition with chronic dosing. We can speculate that interaction with MAO-B might account for the quantitative difference in the efficacy of GSK2 with respect to GSK3 and lamotrigine in the reversal learning model. However, it is unclear the mechanism by which acute MAO-B inhibition might reduce the efficacy of the sodium channel blocker in this test.

A final aim of the present study was to assess the potential of the new sodium channel blockers to produce centrally mediated side-effects typical of their class; for example, sedation, muscle relaxation, and motor incoordination (Upton, et al., 1997). The measurement of an animal's basal activity in a novel environment allows the assessment of these adverse effects (Helton, et al., 1998). In the present study, all three drugs affected vertical activity at lower doses than those that impaired horizontal activity. Vertical activity mainly consists of the animal rearing, and is likely to be a purely exploratory behavior; it may also require greater muscle strength and coordination, and thus may be more sensitive to sedative or ataxic effects of drugs than the general locomotion that contributes to horizontal activity measures. Consistent with this, each of the three drugs produced a significant impairment of vertical activity at lower doses than those that impaired horizontal activity. The

profile of impairment for GSK2 and GSK3 was similar to that of lamotrigine, and consistent with their sodium channel blocking mechanism. As discussed earlier, each drug required a higher plasma concentration for significant efficacy in the reversal learning model compared to the electroshock seizure model, and based on estimates of plasma concentrations required for efficacy in comparison to the measured plasma concentrations associated with motor impairment (Table 1), the therapeutic index for these drugs in patients with schizophrenia may be lower than for patients with epilepsy. However, it should be noted that no impairment of motor behavior (lever pressing) was observed with any dose of the three drugs in the reversal learning study itself.

In conclusion, we have shown that the two novel sodium channels blockers have similar qualitative efficacy to lamotrigine in preventing the cognitive deficits produced by the NMDA receptor antagonist, PCP in a reversal learning task in rats. These results support the evaluation of sodium channel blockers in the treatment of cognitive dysfunction in patients with schizophrenia; however, higher doses than those required for anticonvulsant efficacy may be needed, with a consequently higher risk of mechanism-dependent central side effects.

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Authorship Contributions

Participated in research design: Large, Read, Gunthorpe, Neill, Gozzi, Gill, Alvaro

Conducted experiments: Bison, Sartori, Gozzi, Quarta, Antolini, Hollands, Gill, Idris

Contributed new reagents or analytic tools: Alvaro

Performed data analysis: Large, Quarta, Bison, Antolini, Hollands, Gill, Idris, Gozzi

Wrote or contributed to the writing of the manuscript: Large, Quarta, Gozzi, Gunthorpe, Neill.

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Footnotes

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Figure Legends

Figure 1: The effect of (a) GSK2 (20-80mg/kg, p.o.), (b) GSK3 (10-60mg/kg, p.o.) and (c) lamotrigine (25mg/kg, i.p.) on the deficit produced by acute PCP (1.5mg/kg, i.p.) on performance of the reversal learning task. Data are shown as mean ± S.E.M. of % correct responding (n=10-20 per group). No significant difference between any groups in the initial phase. Significant reduction in percent correct responding of drug treatment groups in the reversal phase compared with the vehicle group; **P<0.01, ***P<0.001; Significant improvement in performance compared to PCP alone in the reversal phase; # P<0.05; ## P<0.01; # ## P<0.001; ANOVA followed by student's t- test.

Figure 2: Anatomical distribution of the rCBV response following PCP administration (0.5 mg/kg i.v., group 5) with respect to baseline (vehicle–vehicle, group 8) as a function of pharmacological pretreatment. Yellow/orange indicate *increased* rCBV versus baseline; blue indicates *decreased* rCBV versus baseline. Z statistics threshold levels are reported beside each map. Maps were cluster-corrected using a p=0.01 significance level. (a) GSK2 (B, C; groups 6 and 7, respectively) or vehicle (A; group 4) were administered s.c. 30 min prior to PCP challenge. (b) GSK3 (C, D; groups 2 and 3, respectively), lamotrigine (B; group 4) or vehicle (A, group 1) were administered i.p. 30 min prior to PCP challenge. Abbreviations: Acb, nucleus accumbens; mPFC, medial prefrontal cortex; Cg; cingulated cortex; Th, thalamus; Rs, retrosplenial cortex; VHc, ventral hippocampus.

Figure 3: Magnitude of mean rCBV response ($AUC_{4\text{-}20min}$) to PCP in representative anatomical volumes of interest. (a) Left: Veh-PCP (group 5); GSK2 30 mg/kg-PCP (group 6). Right: Veh-PCP (group 5); GSK2 100 mg/kg-PCP (group 7); *p<0.05, **p<0.01 versus Veh-PCP, (groups 5), one-way ANOVA followed by Dunnett's test for multiple comparison. (b) Left: Veh-PCP (group 1); GSK3 10 mg/kg-PCP (group 2). Middle: Veh-PCP (group 1); GSK3 30 mg/kg-PCP (group 3); Right: Veh-PCP (group 1); Lamotrigine 10 mg/kg-PCP (group 4); *p<0.05, **p<0.01 versus Veh-PCP, (groups 1), one-way ANOVA followed by Dunnett's test for multiple comparison. Abbreviations: Acb, nucleus

accumbens; Cpu, caudate putamen; DL, dorsolateral thalamus; VM, ventromedial thalamus, AD, anterodorsal hippocampus; V, ventral hippocampus; PDG, posterior dentate gyrus; PD, Posterodorsal hippocampus; S1, primary somatosensory cortex; V1, primary visual cortex; Cg, cingulate cortex; PFC, medial prefrontal cortex.

Figure 4: Effect of GSK2 administered s.c. on PCP-induced elevation of extracellular concentrations of noradrenaline (A), dopamine (B), and serotonin (C) in the rat mPFC. Results represent means ± S.E.M. of percentage of basal values (n=4-8 per group). Basal values were means of three values (-100, -80, -60) before drug administration. Arrows illustrate the drug injection times. Two-way ANOVA was applied for each sequence of treatment with a between-subject factor of treatment and a repeated measures factor of time. Significance of differences between drug treatment (PCP or PCP+GSK2 100mg/kg) and vehicle are indicated as * $p \leq 0.05$; ** $p \leq 0.01$, assessed using a multiple comparison test. Note that significance indicators for the GSK2 50mg/kg group are not included for clarity.

Figure 5: Effect GSK3 administered s.c. on PCP-induced elevation of extracellular concentrations of noradrenaline (A), dopamine (B), and serotonin (C) in the rat mPFC. Results represent means ± S.E.M. of percentage of basal values (n=6-10 per group). Basal values were means of three values (-80, -60, -40) before drug administration. Arrows illustrate the drug injection times. Two-way ANOVA was applied for each sequence of treatment with a between-subject factor of treatment and a repeated measures factor of time. Significance of differences between drug treatment (PCP or PCP+GSK3 30mg/kg) and vehicle are indicated as * $p \leq 0.05$; ** $p \leq 0.01$, assessed using a multiple comparison test.

Figure 6: Effect of drug treatment on vertical and horizontal locomotor behaviour in rats. (a) GSK2 (p.o.) 60 min post-drug, 8 animals per group. (b) GSK3 (s.c.) 60 min post challenge, 9 animals per group. (c) Lamotrigine (p.o.) 90 min post challenge, 9 animals per group. Data are expressed as mean

± S.E.M.; asterisks represent significant differences versus vehicle group detected by ANOVA followed by one tailed Dunnett`s test (*$p < 0.05$; **$p < 0.01$).

Figure 7: Effect of in vivo dosing with GSK2 administered s.c. on ex vivo MAO-B Activity in rat forebrain homogenate. MAO-B activity in forebrain tissues derived from the first (A) and second (B) experiments, 30 min after dosing. Asterisks represent significant differences versus vehicle group detected by one-way ANOVA followed by Post-Hoc Dunnett's Test (* $p<0.05$; ** $p<0.01$).

Table 1: Summary of dose-effect data for the Reversal Learning and LMA studies. Plasma concentrations for the Reversal Learning study were extrapolated from the LMA studies. Plasma concentrations shown are derived from total blood concentration and corrected for plasma protein binding and blood:plasma ratio. Therapeutic index ratios ("fold-Electroshock") are derived from the relative plasmas concentrations. Ki = affinity for the inactivated state of human recombinant $Na_v1.2$ channels; med = minimal statistically significant effect; NOAEL = no adverse event limit (the highest dose tested that did not impair behavior in the test). Data from the rat electroshock seizure model and Nav1.2 assay are from Large et al. (2009).

	NaV1.2	**Electroshock**			**Reversal Learning**			**Motor Behaviour**			
	Ki	med mg/kg	route	free plasma (ng/mL)	med mg/kg	route	free plasma (ng/mL)	NOAEL mg/kg	route	free plasma (ng/mL)	fold-Electroshock
GSK1014802	1.6	3	s.c.	54	60	p.o.	786*	60	p.o.	786	14.6
GSK1061436	0.9	2	s.c.	40	30	p.o.	175*	<30[a]	s.c.	325	<8.1
Lamotrigine	24	3	p.o.	930	25	i.p.	3470*	<3[a]	p.o.	675	<0.7

*data extrapolated from independent pharmacokinetic studies using the same route of administration.

[a] significant effect at this dose on vertical activity; no effect on horizontal activity.

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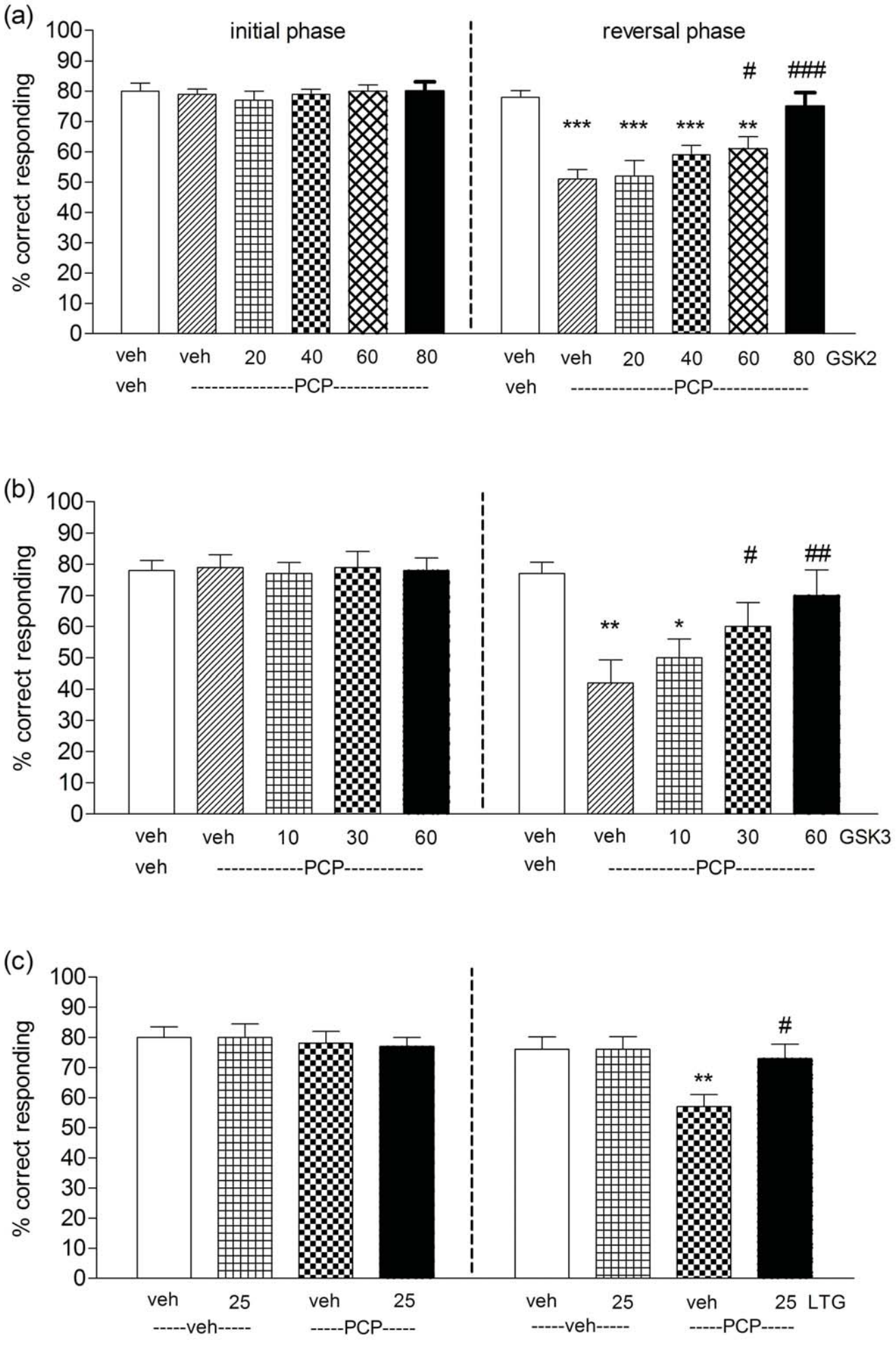
Figure 1



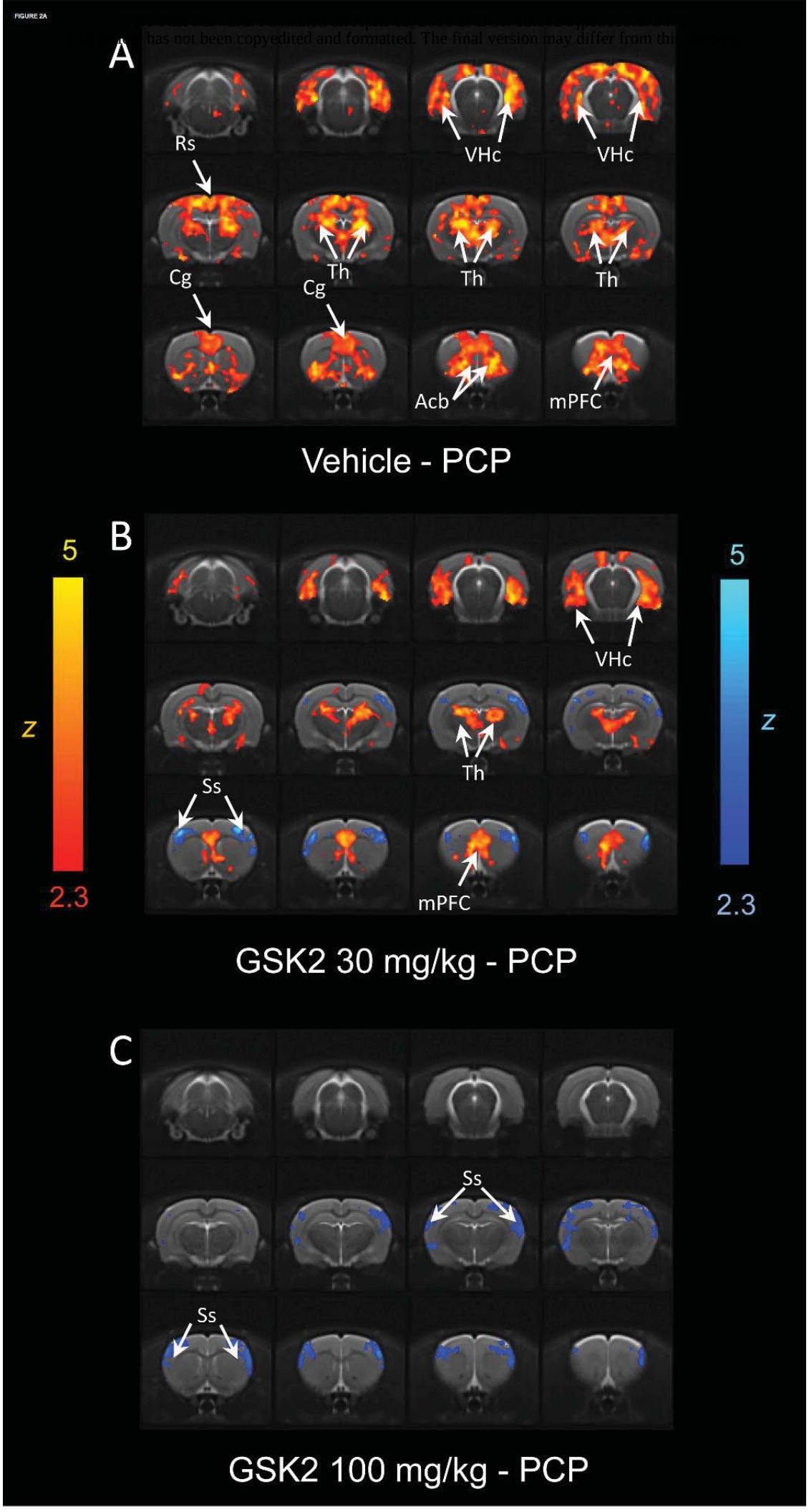


FIGURE 2B

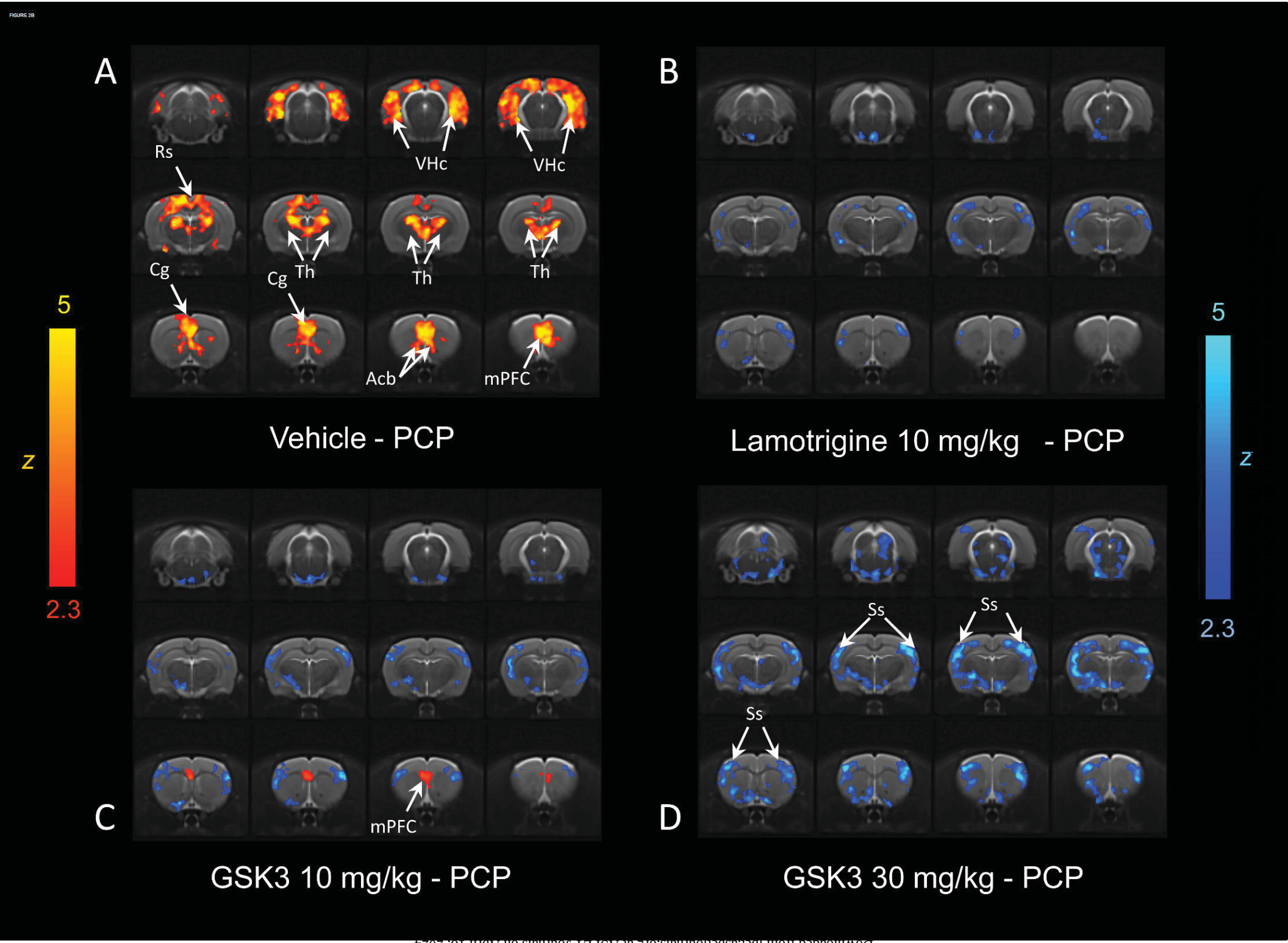
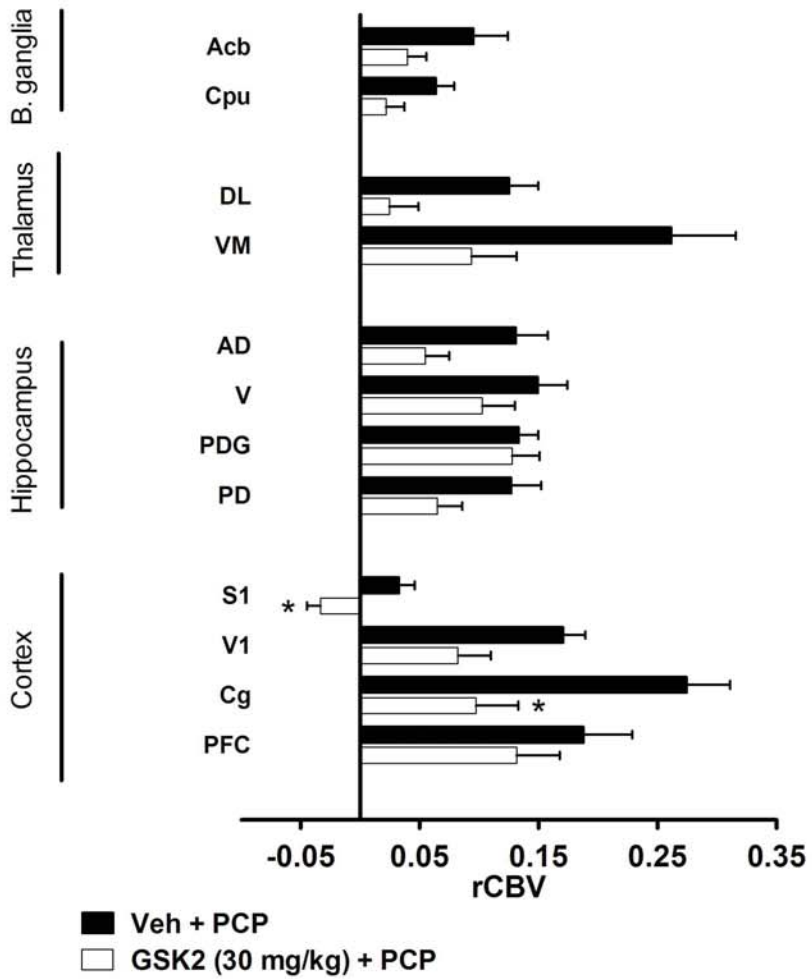


Figure 3a



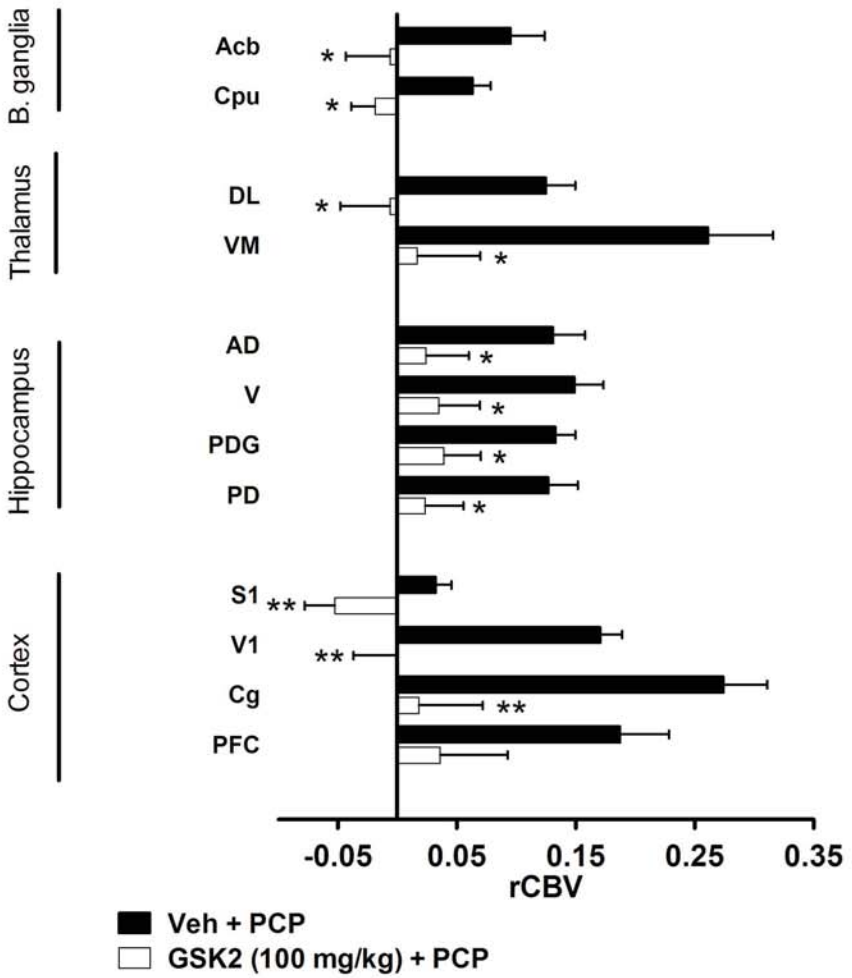


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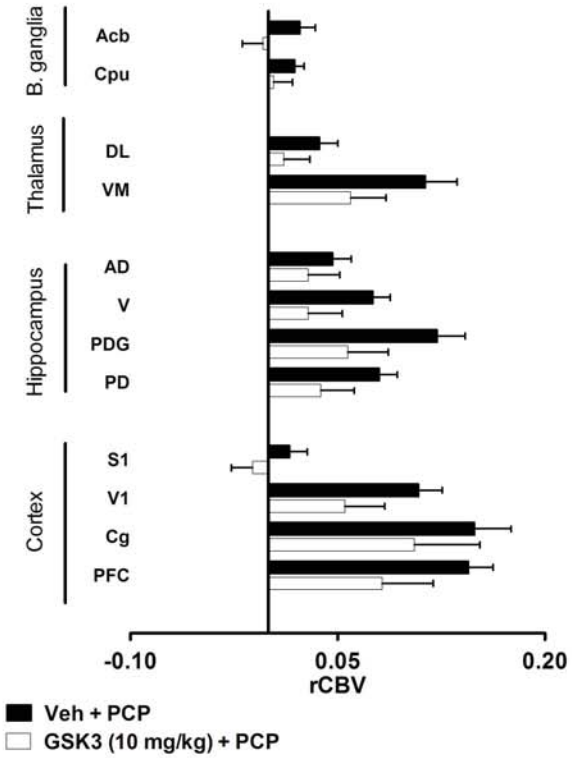
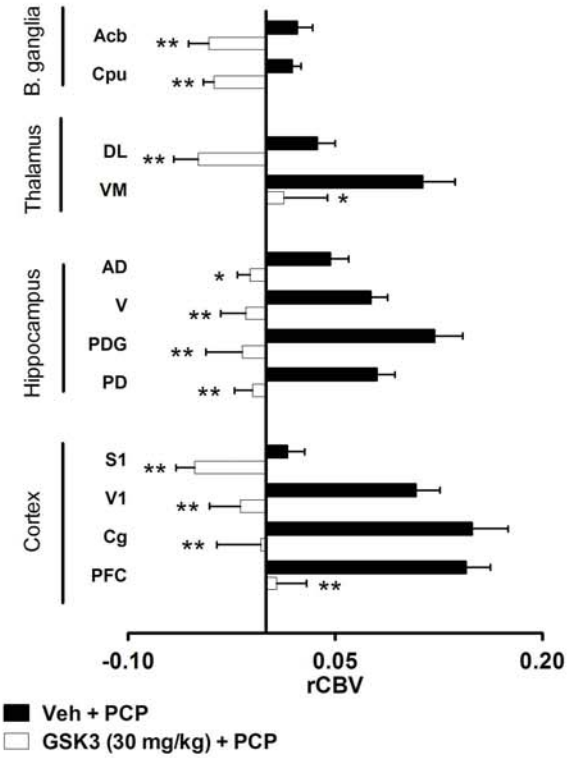
Figure 3b





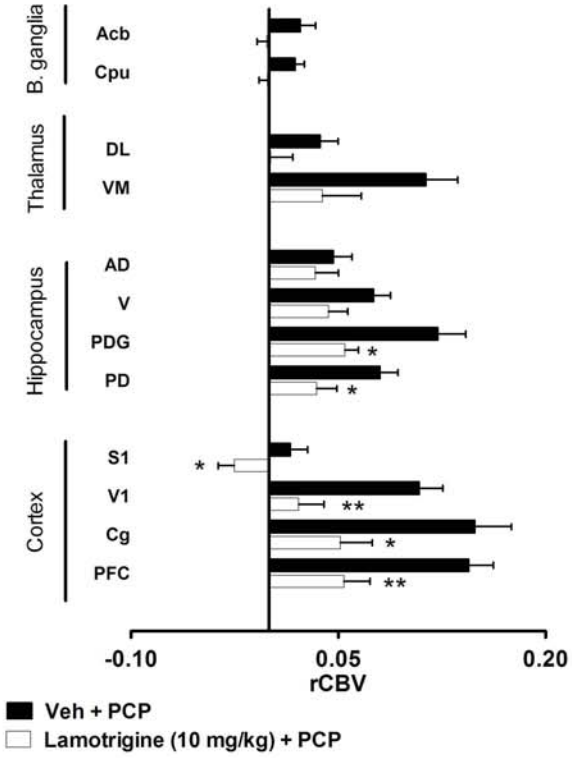


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Figure 4

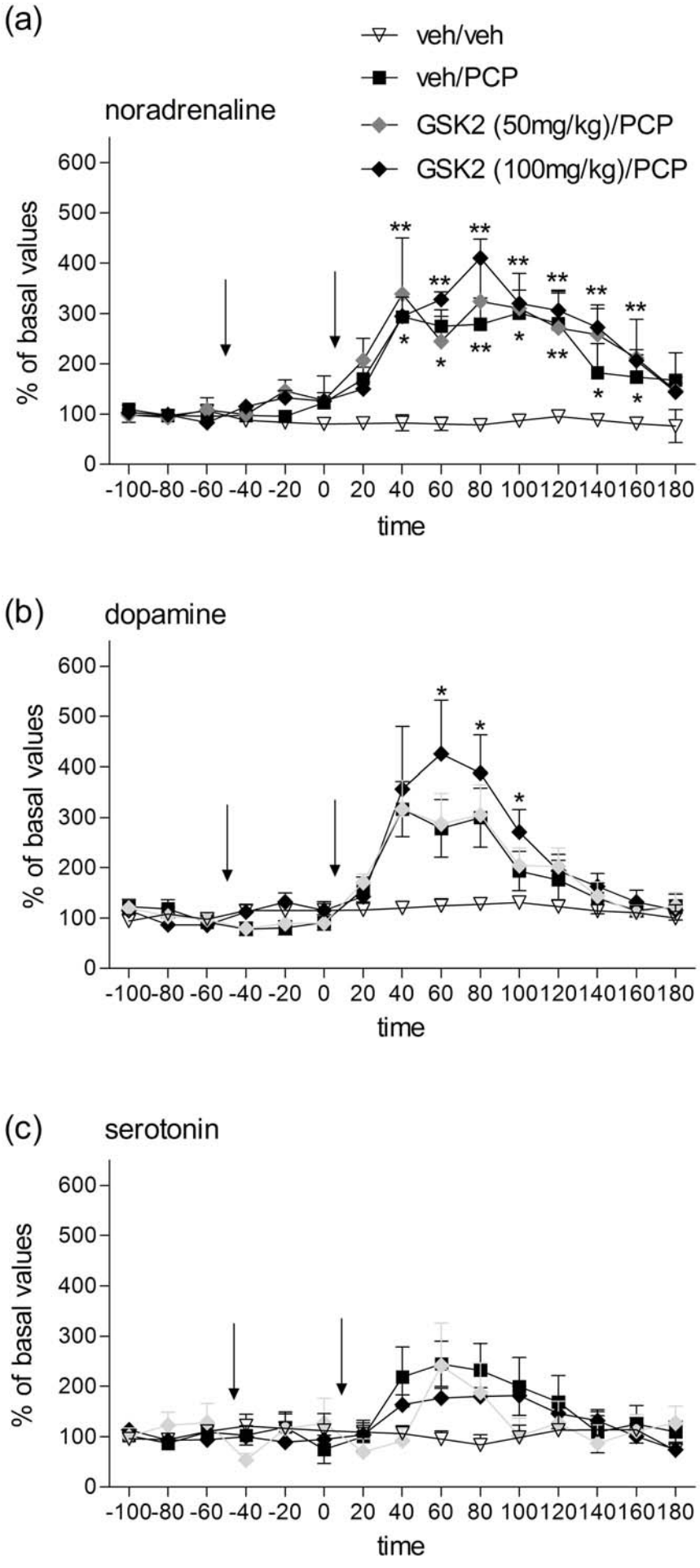

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Figure 5

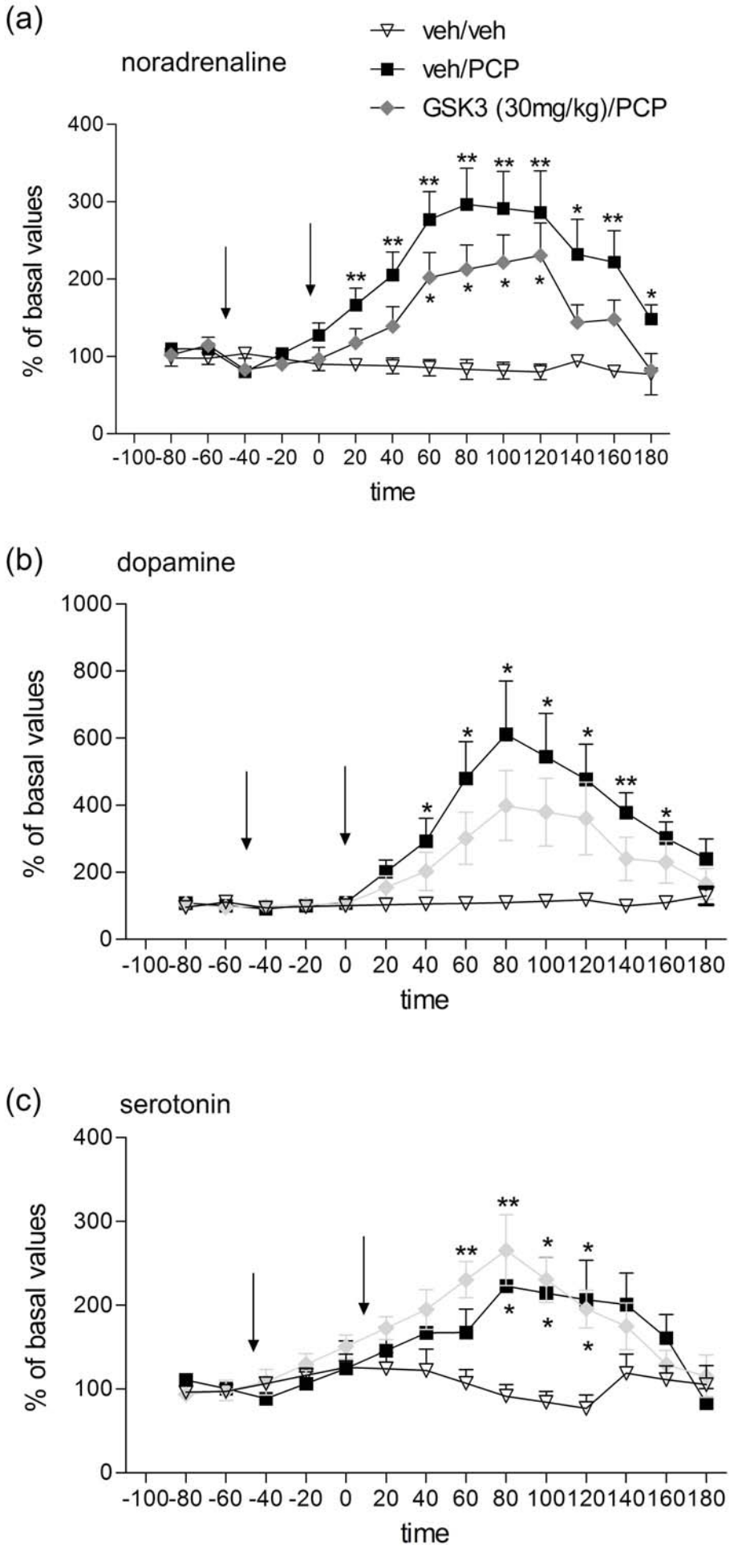

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Figure 6

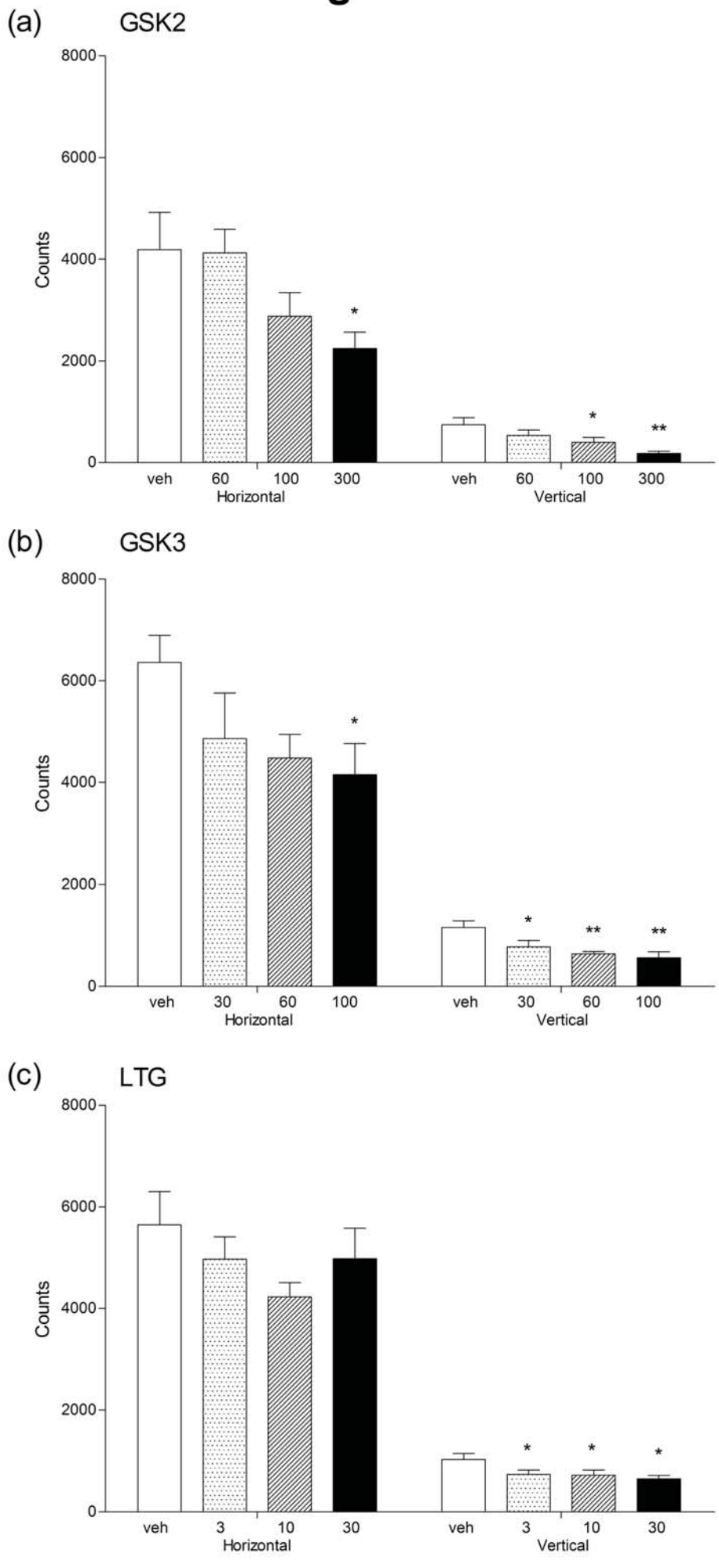

Figure 7