EEG-β/γ spectral power elevation in rat: a translatable biomarker elicited by GABA	extsubscript{Aα2/3}-positive allosteric modulators at nonsedating anxiolytic doses

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Submitted 26 July 2013; accepted in final form 26 August 2014

EEG-β/γ spectral power elevation in rat: a translatable biomarker elicited by GABA	extsubscript{Aα2/3}-positive allosteric modulators at nonsedating anxiolytic doses. J Neurophysiol 113: 116–131, 2015. First published September 24, 2014; doi:10.1152/jn.00539.2013.— Benzodiazepine drugs, through interaction with GABA	extsubscript{Aα1}, GABA	extsubscript{Aα2}, and GABA	extsubscript{Aα5} subunits, modulate cortical network oscillations, as reflected by a complex signature in the EEG power spectrum. Recent drug discovery efforts have developed GABA	extsubscript{Aα2/3}-subunit-selective partial modulators in an effort to dissociate the side effect liabilities from the efficacy imparted by benzodiazepines. Here, we evaluated rat EEG and behavioral end points during dosing of nine chemically distinct compounds that we transfected with those GABA	extsubscript{A} subunits. These compounds were vs. GABA	extsubscript{A} and and 2,3-subunit-selective partial modulators in an effort to dissociate the side effect liabilities from the efficacy imparted by benzodiazepines. Here, we evaluated rat EEG and behavioral end points during dosing of nine chemically distinct compounds that we confirmed statistically to selectively to enhance GABA	extsubscript{A} mediated vs. GABA	extsubscript{Aα1} or GABA	extsubscript{Aα5} currents in voltage clamped oocytes transfected with those GABA	extsubscript{A} subunits. These compounds were shown with in vivo receptor occupancy techniques to competitively displace [3H]flumazenil in multiple brain regions following peripheral administration at increasing doses. Over the same dose range, the compounds all produced dose-dependent EEG spectral power increases in the β- and γ-bands. Finally, the dose range that increased γ-power coincided with that eliciting punished over unpunished responding in a behavioral conflict model of anxiety, indicative of anxiety without sedation. EEG γ-band power increases showed a significant positive correlation to in vitro GABA	extsubscript{Aα2}-modulatory intrinsic activity across the compound set, further supporting a hypothesis that this EEG signature was linked specifically to pharmacological modulation of GABA	extsubscript{Aα2/3} signaling. These findings encourage further evaluation of this EEG signature as a noninvasive clinical translational biomarker that could ultimately facilitate development of GABA	extsubscript{Aα2/3}-subtype-selective drugs for anxiety and potentially other indications. EEG spectral power changes that represent a real-time measure of changing cortical network activity. Such changes can be driven by effects of a drug either directly on local cortical networks, longer projecting thalamocortical circuits, or by modulation of subcortical inputs to cortical networks. EEG spectral changes can qualify as a robust pharmacodynamic biomarker for a drug to engage receptors and regulate network oscillations in the brain circuits expressing those receptors, as reviewed by Leiser et al. (2011). In this regard, benzodiazepine drugs, which modulate GABA signaling nonspecifically at GABA	extsubscript{Aα1}, GABA	extsubscript{Aα2/3}, and GABA	extsubscript{Aα5} subunits, have a well-documented EEG signature. Multiple investigations have demonstrated that benzodiazepine drugs with potent anxiolytic/sedative properties produce robust enhancement of β-band EEG activity in animal models (Coenen and van Luijtelaar 1991; Jongsma et al. 2000; van Lier et al. 2004) and in humans (Saletu et al. 2006; Giles and Luthringer 2007). β-Band elevation has been proposed as a validated quantitative biomarker for GABA	extsubscript{A} receptor modulation with utility for pharmacokinetic/pharmacodynamic modeling (Visser et al. 2003). Under drug-free conditions, EEG β- and γ-band power elevation traditionally has been associated with increased arousal (Brown et al. 2012), as well as higher cognitive functions, such as working memory (Tallon-Baudry et al. 2004) and perception (Rodriguez et al. 1999). The EEG changes produced by benzodiazepine drugs thus present an apparent paradox in that EEG β- and γ-band power enhancement is observed in the face of psychopharmacologically driven overt sedation. This phenomenon has been referred to as “pharmacological dissociation” (Coenen and van Luijtelaar 1991).

Drug discovery programs have developed subtype-selective GABA	extsubscript{A} agents toward the objective of retaining efficacy but reducing the liability profiles endemic to benzodiazepines. This objective emanated initially from a set of observations in genetically engineered mouse models, indicating that anxiolytic and sedative/hypnotic properties of benzodiazepines can be dissociated (Dias et al. 2005; Low et al. 2000; Rudolph et al. 1999; Tobler et al. 2001). Subsequently, this view received partial clinical confirmation with the advent of GABA	extsubscript{Aα1} restricted positive modulators, such as zolpidem (Hoehns and Perry 1993), which act as selective sedative/hypnotics, but not anxiolytics. Conversely, as subtype-selective GABA	extsubscript{Aα2/3}-pos-
positive modulators became available, testing in multiple animal models of anxiety in rodents (Atack et al. 2006; Dias et al. 2005; McKernan et al. 2000; Morris et al. 2006) and primates (Atack et al. 2006) has demonstrated anxiolytic efficacy in the absence of sedation and cognitive impairment. This was confirmed clinically with development drug candidate, TPA023 (Atack 2009; Carling et al. 2005; de Haas et al. 2007). However, there remain major unresolved questions in the successful development of GABA_{A_{2/3}} agents as drugs, a key one concerning the optimal fine tuning of positive modulatory efficacy needed to maximize anxiolytic or other therapeutic benefits, while minimizing side effect liabilities (Atack et al. 2009).

The spectral EEG signature of GABA_{A_{2/3}}-subunit-selective modulators has been deduced indirectly by loss of function studies in genetically engineered mice in which the GABA_{A_3} (Kopp et al. 2003) or GABA_{A_2} (Kopp et al. 2004) benzodiazepine binding site was rendered insensitive. Recently, Nickolls et al. (2011) evaluated the EEG signature of TPA023 and a second higher efficacy GABA_{A_{2/3}-positive} subtype-selective positive modulator, L-838,417. They found that L-838,417, but not TPA023, elicits a dose-dependent increase in EEG β-band power. This raised the possibility that these EEG changes could represent a useful real-time biomarker if pharmacologically specific could be associated more conclusively with GABA_{A_{2/3}}-positive modulation. Establishing such a connection in animal studies would, in turn, enable a translational hypothesis highlighting utility of these EEG changes as a pharmacodynamic biomarker to facilitate clinical development of these agents. Encouraged by this idea, we designed the current experiments to evaluate systematically the EEG signature for a chemically diverse collection of selective GABA_{A_{2/3}} central nervous system (CNS) penetrant research compounds, and in tandem determine their efficacy in a conflict model of anxiety (Treit 1985) to evaluate correspondence in the dose-response relationships between the EEG changes and predicted efficacy dose. To stabilize behavioral and concurrently monitor for sedation during EEG collection, rats continuously performed an operant task during recordings. The studied compound set included the literature compound, TPA023 discussed above, the literature compound TP003, which is a putative GABA_{A_{1-3}}-subtype-selective modulator (Dias et al. 2005), and seven novel, subtype-selective GABA_{A_{2/3}} modulators discovered at AstraZeneca. Two of these latter molecules, AZD6280 and AZD7325, were advanced to clinical trials. The drugs, lorazepam (nonselective positive modulation via GABA_{A_{1-2,3,5}} subtypes) and zolpidem (selective positive modulation via GABA_{A_{1}} subtype) were also incorporated in the study as reference comparators.

Our results revealed that the GABA_{A_{2/3}}-selective compounds all shared a dose-dependent EEG spectral signature comprised of β/γ-band power elevation, and this overlapped their anxiolytic dose range. These findings are discussed in the context of a mechanism-specific translational EEG biomarker that can be exploited to facilitate future development of GABA_{A_{2/3}} subtype-selective compounds for a range of proposed disease indications, including anxiety, and chronic pain. Some findings reported here have been published previously in abstract form (Christian et al. 2008).

MATERIALS AND METHODS

Drugs and compounds. Lorazepam and zolpidem were obtained from the AstraZeneca Pharmacy and formulated in either a solution of 40% propylene glycol, 10% ethanol, and 50% H2O at 2 mg/ml stock concentration for the behavioral conflict studies, or 20% (wt/vol) sulphobutylether-β-cyclodextrin (SBECD; pH: 6.0–6.5) vehicle at stock concentrations of 2 mg/ml for EEG studies. Serial dilutions were made with these same buffers to desired doses for subcutaneous injection at a dosing volume of 1 ml/kg. TP003 and TPA023 were synthesized at AstraZeneca and formulated in 20% SBECD (pH: 2.5) at 30 μmol/ml initial concentration and then further diluted in SBECD to the indicated doses. Seven novel putative GABA_{A_{2/3}}-subtype-selective agents synthesized de novo at AstraZeneca were profiled in the present study. The synthesis, structure and pharmacological properties of each of these compounds is described by Alhambra et al. (2011). Five of the seven compounds are identified here by a unique compound number that cross-references directly to numbers designated for these compounds in Tables 3–7 of Alhambra et al. (2011) [i.e., compounds (Cmpd) 29, 30, 43, 44, and 45]. The final two AstraZeneca development compounds, AZD6280 and AZD7325, cross reference, respectively, to Cmip 13 and 40 in Alhambra et al. (2011).

Evaluation of GABA_{A_{1-2,3,5}}-positive modulatory activity by two-electrode voltage clamp. Recordings were made from Xenopus laevis oocytes injected with cRNA of GABA_{A} β_2/3-subtype selectivity. Establishing such a connection in animal studies would, in turn, enable a translational hypothesis highlighting utility of these EEG changes as a pharmacodynamic biomarker to facilitate clinical development of these agents. Encouraged by this idea, we designed the current experiments to evaluate systematically the EEG signature for a chemically diverse collection of selective GABA_{A_{2/3}} central nervous system (CNS) penetrant research compounds, and in tandem determine their efficacy in a conflict model of anxiety (Treit 1985) to evaluate correspondence in the dose-response relationships between the EEG changes and predicted efficacy dose. To stabilize behavioral state and concurrently monitor for sedation during EEG collection, rats continuously performed an operant task during recordings. The studied compound set included the literature compound, TPA023 discussed above, the literature compound TP003, which is a putative GABA_{A_{1-3}}-subtype-selective modulator (Dias et al. 2005), and seven novel, subtype-selective GABA_{A_{2/3}} modulators discovered at AstraZeneca. Two of these latter molecules, AZD6280 and AZD7325, were advanced to clinical trials. The drugs, lorazepam (nonselective positive modulation via GABA_{A_{1-2,3,5}} subtypes) and zolpidem (selective positive modulation via GABA_{A_{1}} subtype) were also incorporated in the study as reference comparators.

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J Neurophysiol • doi:10.1152/jn.00539.2013 • www.jn.org
All aspects of the in vivo protocol were conducted as for the AZD7325, TPA023, Cmpd 43 and Cmpd 44 using unlabeled flumazenil (5.0 μCi/ml–1 rat–1 iv; equivalent to 0.062 nmol dose). Rats were euthanized 5 min after radioligand injection, the brain removed and the frontal cortex (FC) and pons regions isolated. Tissue was weighed and then solubilized overnight in the presence of Soluene 350. Ultima-Gold scintillation fluid was added, and total radioactivity was measured using a Tri-Carb scintillation counter (Perkin Elmer).

$[^3H]$Flumazenil binding was determined for all treatment groups and represented as fmol/mg tissue. Specific binding (SB) was calculated from total binding values: the fmol/mg value for the pons (region of nonspecific receptor binding) was subtracted from the target region, FC fmol/mg value. Two-way ANOVA with Bonferroni post hoc analysis was used to determine the significance of the SB values. In vivo receptor occupancy for each pretreatment compound was indicated by a reduction in SB of $[^3H]$Flumazenil and compared with vehicle controls and represented as a percentage using the following equation:

$$\%RO = 100 \times \frac{(SB_{\text{saline}} - SB_{\text{test compound}})}{SB_{\text{saline}}}$$

where $\%RO$ is percent receptor occupancy. Occupancy curves were fit (sigmoidal curve fit with variable slope), and nonlinear regression analysis was used to calculate the ED$_{50}$ and ED$_{80}$ values using Prism GraphPad (GraphPad, San Diego, CA).

In vivo receptor occupancy was alternatively measured for AZD7325, TPA023, Cmpd 43 and Cmpd 44 using unlabeled flumazenil (0.062 nmol/rat iv) by the LC/MS (high performance liquid chromatography combined with triple quadrupole mass spectral method). All aspects of the in vivo protocol were conducted as for the $[^3H]$Flumazenil experiments. For the LC/MS method, frozen tissue from the FC was homogenized in two volumes of CH$_3$CN/H$_2$O (80:20) and centrifuged at 13,000 rpm for 15 min. An Agilent 6410 Triple Quadrupole LC/MS equipped with an electrospray ionization source and coupled to an Agilent 1200 Series Rapid Resolution LC system was used. Known concentrations of $[^3H]$Flumazenil in drug-treated samples was referenced against $[^3H]$Flumazenil in drug-treated samples. Rats were fed and watered ad libitum prior to and following pretreatment for each compound, and plasma concentration measurements to determine occupancy value.

To monitor exposure, trunk blood samples were collected at 35 min following pretreatment for each compound, and plasma concentration determined by LC/MS using electrospay in the positive ionization mode. Plasma proteins were precipitated by the addition of 0.1% formic acid in acetonitrile/0.1% formic acid in water (80:20). The supernatants were transferred for analysis by reverse-phase gradient elution LC/MS.

In vivo electrophysiology. Sprague-Dawley rats (360–430 g) served as subjects. Rats were fed and watered ad libitum prior to and for ≥10-day recovery period following surgery. Rats subsequently were food restricted for the duration of the study to maintain their postrecovery weight within ~5% for behavioral training purposes. For surgery, rats were anesthetized with inhalational isoflurane (2–5% in O$_2$). Body temperature was maintained at 37°C with a homeothermic blanket. Rats were surgically instrumented for skull and stored on a desktop computer.

Prior to recording, rats were trained to criterion on a single tone auditory operant discrimination paradigm in the operant chamber. The chamber was housed within a larger closed opaque acoustical chamber, in which rats were monitored by a video camera. The operant paradigm was computer controlled and delivered through a MED-SYST-8 interface (Med Associates) that also tracked performance statistics achieved by animals in the paradigm. A nose-poke response receptor containing an infrared photocell beam and detector was mounted 1.12 cm above the floor bar grid on one side wall. A 2 in. width × 6 in. height × 0.75 in. depth recessed pellet receptacle was located on the opposite wall. Feed pellets (45 mg) were dispensed from a magazine into this receptacle for consumption by the rat. A speaker and house light were mounted on the side walls near the top of the chamber. The operant task consisted of repetitive tone (1 kHz, 500-ms duration, ~70–dB at center of chamber) presentations at a random intrastimulus interval of 28–38 s, delivered through the speaker from a programmable audio generator (Med Associates). Responses (nose-poke breaks of the photoreceptacle beam) within 5 s of tone onset were termed “correct” and rewarded by immediate dispensation of a food pellet. All responses outside of this 5-s window were termed “incorrect” and were unrewarded. Animals reached stable criterion performance (correct responses made to >80% of tones, and comprising >30% total responses) within ~10 daily 1-h training sessions once they attained the instrumental association to the tone.

The drug compound testing protocol consisted first of acclimating an animal to the chamber for 10–20 min. Tones were presented until three or more correct responses were made. A continuous EEG recording during a 30-min baseline epoch was then obtained, as the animal continually performed the operant task. Subsequently, the animal was briefly disconnected at the commutator, removed from the chamber, dosed either by the per os or subcutaneous route as specified for each compound (or equivalent volume of vehicle), and then reintroduced to the chamber and the recording/behavioral protocol immediately reintiated for a 30-min postdose block. This dosing procedure typically spanned <2 min with little disruption to the animal. The animal was then subjected to the same procedural cycle for each subsequent ascending compound dose. In experiments excepting those with both of the compounds, the animal received three to four ascending doses of the test compound, or successive corresponding vehicle doses, resulting in a total recording time of 2–2.5 h (i.e., 30 min predosing + 90–120 min postdosing 3–4 doses of each compound). Cmpd 43 and 44, in contrast, were administered at a single high dose following a 30-min baseline recording. Subsequent EEG was then recorded continuously for 1.5 h.

Following a recording session, animals were subjected to a washout period >1 wk before being recorded again. During this washout, animals were presented with intermittent training in the operant paradigm, sufficient to sustain criterion performance. Drug and vehicle experiments were randomized in all animals. Each of the animals contributed one to two replicates to the total data set for a given treatment and one paired vehicle experiment.

EEG data were analyzed using NeuroExplorer version 3.183 software (Plexon). A time series of fast Fourier transforms (step size: 10 s) was applied, and EEG power density computed from 1–50 Hz with a resolution of 0.068 Hz for each transform. The EEG spectral power was partitioned into bands in accordance with the International Phact
macological EEG Group Guidelines (see Versavel et al. 1995), as follows: \( \delta \), 1–5.5 Hz; \( \theta \), 5.5–8.5 Hz; \( \alpha \), 8.5–12.5 Hz; \( \beta \), 12.5–30 Hz; \( \gamma \), 30–50 Hz without further subbanding the \( \alpha \)- and \( \beta \)-bands. For analysis of drug effects, the average power spectral density within each band was determined for the 20-min block just prior to vehicle or drug dosing (i.e., \( -20 \) to \( 0 \) min), and then for a 20-min postdosing period commencing \( 10 \) min after dosing (i.e., \( +10 \) to \( +30 \) min postdosing). The 10-min delay following dosing was judged sufficient to allow adequate CNS exposure for effects on the EEG to achieve a plateau, based on independent pharmacokinetic evaluation of \( r_{\text{max}} \) for the compounds (not shown). For comparison within and across treatments and dose levels, the averaged EEG power density in each band for each 20-min postdosing block in an experiment was normalized to the average power during the 20-min predosing block.

Behavioral performance data acquired by the Med Associates interface (%correct responses to total tones; ratio of correct/nonrewarded responses; mean response latency from tone onset to operant nose-poke response) were also determined for each treatment block within an experiment and compared with the predosing values across experiments and treatments.

Normalized power spectral density data for each compound dose in each designated EEG band were evaluated initially by two-way ANOVA on factors of 3 dose levels \( \times \) 2 (vehicle and compound) treatment levels. Significant main effects were assessed further between possible pairwise comparisons using the Bonferroni individual comparisons method. A value of \( P < 0.05 \) was taken to denote a significant effect in all statistical tests. A second set of two-way ANOVAs was initially performed for vehicle, lorazepam, AZD6280 and AZD7325 with factors of 3 dose levels \( \times \) 2 recording sites (frontal vs. temporal). However, no significant differences emerged in any case for the recording site factor. Subsequent analyses for these and other compounds shown in figures are considered using data from the active frontal recording lead only.

**Rat conflict behavioral model for anxiety.** Male Long Evans rats (350–500 g) were used for all studies. Rats were food-restricted to 85% of free feeding weight. A standard two-lever operant chamber (Med Associates) contained two retractable response levers, each with a stimulus lamp above it. A house light was mounted on the back wall of the chamber. Forty-five milligram food pellets were delivered to a cup mounted below and between the two response levers. The grid floor of the chamber was interfaced to shock generators. All events in the chambers were controlled and monitored by a microprocessor.

The behavioral task was composed of two components: 1) unsuppressed responding (unpunished) for 2-min duration; 2) suppressed responding (punished) for 3-min duration. In the unpunished component, the house light and both stimulus lamps over the response levers were turned on, the lever on the left-hand side of the chamber extended, and a food pellet was delivered following an average of 17 responses (variable ratio 17 schedule; VR17) on the lever in the chamber (range 3–40 responses). In the punished component, the right-hand lever was extended into the chamber, and the stimulus lamps and houselights were turned on and off at 1-s intervals, in succession, which served as a cue. Food was available under the same VR17 schedule as in the unpunished component, but, in addition, electrical current (0.5-s duration) was delivered to the grid floor of the chamber under an independent VR17 schedule. The level of the current was adjusted (0.2 mA to 0.75 mA) for each individual rat until responding was suppressed to a level \( -5\%\)–\( -10\% \) that of the unpunished component. Alternating unpunished and punished components (5 of each) were separated by 10-s time-out periods in which both response levers were retracted and all stimulus lamps turned off. Daily sessions always began with the unpunished responding component, and consisted of five cycles, each encompassing an unpunished (2 min each) and punished component (3 min each). For any given compound test, rats responding at a stable rate (defined as baseline responding that did not significantly vary for \( \geq 3 \) days over 1 wk, including 1 vehicle administration day) were chosen from a larger pool of trained rats. Several doses were tested randomly on a given test day in different subjects. Each dose was tested subsequently in a different subset of rats. The dependent variables recorded were the rate of responding in unpunished and punished components (total responses/total time under the component), and the number of shocks delivered.

Absolute rate of responding in punished and unpunished components was measured for individual subjects. The percent control rate of responding was then calculated for each individual subject by the following formula:

\[
\text{(rate of responding following compound/rate following vehicle)} \times 100
\]

The Student t-test was used to compare mean control rates for a given set of rats to their corresponding rate of responding after compound administration. A selective anxiolytic effect was defined as an increase in responding in the punished components with no significant effect on responding in unpunished components.

**RESULTS**

Development of diverse chemical structures with partial positive modulatory efficacy and selectivity at GABA\(_{A_{123}}\) subunits. The GABA\(_{A_{123}}\)-subtype-selective modulator, TPA023 (Atack et al. 2006), and the putative GABA\(_{A_{13}}\)-subtype-selective modulator, TP003 (Dias et al. 2005), were discovered by Merck Pharmaceuticals and have been widely described pharmacologically. In addition to subtype selectivity, these compounds also exhibit low maximal efficacy for modulating GABA signaling, relative to the nonselective benzodiazepine drugs (see Atack et al. 2006).

GABA\(_{A_{123}}\)-subtype selectivity for TPA023, for the putative GABA\(_{A_{13}}\)-subtype-selective modulator, TP003, and each of the seven AstraZeneca internal compounds was ascertained here by concentration-response characterization on GABA current responses mediated via human recombinant GABA\(_{A_{11}}\), GABA\(_{A_{12}}\), GABA\(_{A_{21}}\) and GABA\(_{A_{55}}\) receptors, each heterologously reconstituted with GABA\(_{A_{21}}\) and GABA\(_{A_{22}}\) accessory subunits in Xenopus oocytes. Percent maximal potentiation of GABA EC\(_{10}\) current was established by exposing oocytes to increasing concentrations of a compound until an apparently saturating response was reached. The extent of potentiation of the GABA EC\(_{10}\) current was determined for each concentration, as well as the percent potentiation normalized to that elicited by a supramaximal concentration of the benzodiazepine benchmark, diazepam. Figure 1 shows the concentration-response curves at each of the GABA\(_{A_{21}}\) subtypes for the literature compound, TPA023, and the two AstraZeneca clinical candidate compounds, AZD6280 and AZD7325. Note that the concentration-response curves of GABA current potentiation at GABA\(_{A_{12}}\) or GABA\(_{A_{55}}\) containing receptors approached an asymptotic maximal response in the nanomolar to low micromolar dose range. In contrast, the compounds exerted minimal effects on GABA currents in oocytes containing receptors expressed with GABA\(_{A_{11}}\) or GABA\(_{A_{55}}\) subunits over these same concentration ranges. This selectivity was confirmed statistically (Table 1 and below). In addition to the high functional subtype selectivity for GABA\(_{A_{12}}\) and GABA\(_{A_{13}}\)-containing receptors, the GABA currents induced by all three compounds produced notably less maximal potentiation at these subtypes (\( \sim 15\%–40\% \)) than that achieved by a supramaximal concentration of diazepam. This pharmacologi-
The five additional AstraZeneca compounds in the set were likewise tested at heterologously expressed GABA\(_\text{A}\) receptors in oocytes (Table 1) and conformed generally to a pharmacological profile analogous to the compounds in Fig. 1. All of the compounds shared high functional selectivity for GABA\(_\text{A}\)/\(\alpha_2/\alpha_3\)-containing channels vs. GABA\(_\text{A}\)/\(\alpha_1\)-containing channels, as confirmed statistically. In addition, all of the compounds except Cmpd 30 and 43 showed significant functional selectivity for GABA\(_\text{A}\)/\(\alpha_2/\alpha_3\) over GABA\(_\text{A}\)/\(\alpha_5\)-containing channels. Additionally, Cmpd 30 and 43 and TPA023 were noted to show significant GABA\(_\text{A}\)/\(\alpha_1\) over GABA\(_\text{A}\)/\(\alpha_5\) Selectivity. Finally, the nine compounds spanned a continuum in the degree to which a 1 \(\mu\)M concentration increased GABA EC\(_{10}\) current through GABA\(_\text{A}\)/\(\alpha_2\) or GABA\(_\text{A}\)/\(\alpha_3\) channels from 11–53%, relative to the modulatory effect elicited by a supramaximal diazepam concentration.

For three of the AstraZeneca compounds, including the two clinical candidates and Cmpd 43, pharmacology apart from GABA\(_\text{A}\) channels was investigated more broadly. These compounds were evaluated by binding assays at a 10 \(\mu\)M concentration across a set of 130 targets, including ion channels, G protein-coupled receptors, enzymes and transporters (MDS Panlabs, Bothell, WA). Binding activity across this panel was negligible apart from expected high affinity at GABA\(_\text{A}\) channels, and for the two clinical candidates, displacement at melatonin type 1 and 2 (MT1, MT2) receptors. A guanosine 5’-O-(3-thiotriphosphate) functional assay further revealed that the two clinical candidate compounds showed agonist activity.

Fig. 1. Positive modulation of GABA currents (\(I_{\text{GABA}}\)) at reconstituted human GABA\(_\text{A}\) channels composed of \(\beta_3\), \(\gamma_2\), and either \(\alpha_1\), \(\alpha_2\), \(\alpha_3\), or \(\alpha_5\)-subunits by exemplar GABA\(_\text{A}\)/\(\alpha_2/\alpha_3\)-subtype-selective compounds. Currents were recorded in response to a GABA EC\(_{10}\) concentration from *Xenopus* oocytes with the transiently expressed \(\alpha\)-subunits, as indicated in the presence of the increasing concentrations of TPA023 (A), AZD6280 (B), or AZD7325 (C), as indicated on the \(x\)-axis of each plot. Left: mean (±SE) plateau currents normalized as a percentage of the control current prior to compound exposure. Right: mean (±SE) currents normalized relative to the current potentiation produced by 1 \(\mu\)M diazepam, which elicited a near maximal response. Solid lines represent iterative sigmoidal dose-response fits to each data set. Note that all of the compounds produced a dose-dependent selective potentiation at GABA\(_\text{A}\)/\(\alpha_2/\alpha_3\) and GABA\(_\text{A}\)/\(\alpha_1\) subunits with relatively little effect at GABA\(_\text{A}\)/\(\alpha_2/\alpha_3\) and GABA\(_\text{A}\)/\(\alpha_5\) subunits, and that the maximal effects achieved via GABA\(_\text{A}\)/\(\alpha_2/\alpha_3\) and GABA\(_\text{A}\)/\(\alpha_1\) subunits were partial, relative to the non-selective full modulator, diazepam.
at MT1 receptors in the nanomolar range. However, this feature was not shared by Cmpd 43, which exhibited binding activity for MT1 in the micromolar range, yet insignificant agonist activity. Thus the strongly overlapping EEG signatures and behavioral effects in the anxiety model common to all of these compounds (see below) would be difficult to reconcile as being driven by MT1, as opposed to GABA_{A_α2/3} pharmacology.

Our findings failed to replicate the high functional selectivity of TP003 restricted to channels containing the GABA_{A_α2} subunit, as established by Dias et al. (2005). We do not have a definitive explanation to account for this discrepancy. A methodological comparison establishes that we tested reconstituted channels containing the same accessory subunits (β3, γ2) as those investigators. One obvious difference in the methodologies is that Dias et al. employed a combination of transient and stable transfection methods in mammalian cell lines, as opposed to our transient oocyte transfections. Possibly, differences in subunit expression ratios, density of expression or intracellular regulatory factors could have led to the discrepancy between the studies, although we cannot offer substantive evidence to support any of these possibilities.

CNS bioavailability of GABA_{A_α2/3}-selective agents as assessed by in vivo receptor occupancy. The biodistribution, pharmacological selectivity and receptor-specific regional localization of the GABA_{A_α1,2,3,5} radioligand, [3H]flumazenil, in the rat brain following intravenous administration was validated in house prior to the current study, and aligned with previously reported findings for [3H]flumazenil (Benavides et al. 1992; Goeders and Kuhar 1985). All AstraZeneca compounds excluding Cmpd 30 and 44 were administered to rats over an increasing dose range, and specific displacement of [3H]flumazenil was evaluated. The dose-dependent displacement of flumazenil for all tested compounds trended toward saturation. Figure 2A shows calculated receptor occupancy vs. dose for the two AstraZeneca clinical candidates, AZD6280 and AZD7325. The AZD6280 occupancy curve and ED_{50} showed a right shift in affinity compared with the occupancy

Table 1. Normalized potentiation of GABA-mediated currents produced by GABA_{A_α2/3}-selective study compounds in oocytes expressing various GABA_{A_α} subunits

<table>
<thead>
<tr>
<th>Subunit</th>
<th>α1</th>
<th>α2</th>
<th>α3</th>
<th>α5</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZD6280</td>
<td>14 ± 6 (4)**</td>
<td>91 ± 7 (11)</td>
<td>141 ± 12 (11)</td>
<td>16 ± 5 (4)**</td>
</tr>
<tr>
<td>%DZ</td>
<td>9 ± 3</td>
<td>36 ± 4</td>
<td>34 ± 3</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>AZD7325</td>
<td>-1 ± 2 (20)**</td>
<td>50 ± 3 (19)</td>
<td>47 ± 3 (10)</td>
<td>21 ± 3 (9)**</td>
</tr>
<tr>
<td>%DZ</td>
<td>0 ± 1</td>
<td>19 ± 1</td>
<td>17 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Cmpd 29</td>
<td>2 ± 3 (11)**</td>
<td>72 ± 5 (8)</td>
<td>92 ± 5 (11)</td>
<td>29 ± 3 (5)**</td>
</tr>
<tr>
<td>%DZ</td>
<td>1 ± 1</td>
<td>25 ± 1</td>
<td>19 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
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<td>58 ± 8 (5)</td>
<td>13 ± 7 (6)</td>
</tr>
<tr>
<td>%DZ</td>
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<td>13 ± 3</td>
<td>19 ± 3</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Cmpd 43</td>
<td>-1 ± 2 (23)**</td>
<td>62 ± 4 (15)</td>
<td>95 ± 4 (23)</td>
<td>96 ± 3 (24)</td>
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<tr>
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<td>140 ± 5 (10)</td>
<td>186 ± 15 (6)</td>
<td>71 ± 7 (6)**</td>
</tr>
<tr>
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<td>3 ± 2</td>
<td>53 ± 3</td>
<td>52 ± 2</td>
<td>34 ± 3</td>
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<td>12 ± 3 (14)**</td>
<td>114 ± 6 (10)</td>
<td>140 ± 9 (5)</td>
<td>14 ± 6 (5)**</td>
</tr>
<tr>
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<td>40 ± 2</td>
<td>7 ± 3</td>
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<td>39 ± 6 (9)</td>
<td>36 ± 6 (5)</td>
<td>40 ± 7 (3)</td>
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<td>57 ± 3 (7)</td>
<td>14 ± 10 (4)</td>
</tr>
<tr>
<td>%DZ</td>
<td>5 ± 3</td>
<td>15 ± 2</td>
<td>17 ± 1</td>
<td>6 ± 4</td>
</tr>
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</table>

%Effect denotes for each compound at 1 μM test concentration the positive modulation in GABA membrane current expressed as a mean percent increase (±SE, and n = no. of oocytes tested) of the control current elicited by a GABA EC_{10} concentration in absence of the modulator. GABA_{A_α1} and GABA_{A_α5} currents that are significantly smaller than GABA_{A_α2} current are noted (ANOVA, Bonferroni multiple comparisons; *P < 0.05, **P < 0.01, ***P < 0.001). GABA_{A_α2} and GABA_{A_α5} currents did not significantly differ for listed compounds, with the exception that GABA_{A_α2} significantly exceeded GABA_{A_α5} in case of AZD6280 (P < 0.01), compound (Cmpd) 30 (P < 0.05), Cmpd 43 (P < 0.001), and TPA023 (P < 0.05). %DZ denote for each compound at 1 μM test concentration the positive modulatory effect on membrane current to a GABA EC_{10} concentration normalized as a percentage (±SE) of that produced by a supramaximal concentration (1 μM) of diazepam.

Fig. 2. Percent in vivo specific receptor occupancy in frontal cortex following peripheral subcutaneous (sc) administration of study exemplar GABA_{A_α2/3} compounds at ascending doses. A: calculated mean (±SE; n = 15–18 rats) percent receptor occupancy measured to indicated doses of AZD6280 (Cmpd #13) and AZD7325 (Cmpd #40) by displacement of the radioligand, [3H]flumazenil (solid fits) and comparably for AZD7325 by unlabeled flumazenil, as measured by the LC/MS method, high-performance liquid chromatography combined with triple quad mass spectral analysis (dashed fit; see MATERIALS AND METHODS). B: calculated mean (±SE; n = 6) receptor occupancy measured to indicated doses of TPA023 and compound (Cmpd) #44 using unlabeled flumazenil and the LC/MS method. Best fits of data sets as shown were obtained by iterative fitting to a sigmoidal dose-response function. Note the two different methods used to determine occupancy produced comparable results for AZD7325 (A), and the LC/MS method produced results for TPA023 (B) comparable to those demonstrated in the literature for this compound with the radioligand. [3H]flumazenil (see DISCUSSION).
curve and ED50 for AZD7325, potentially indicative of the slight difference in GABA subtype selectivity, as measured functionally in oocytes. Note that AZD7325 was additionally tested using the flumazenil LC/MS protocol (dashed line), and a less than onefold difference was found between this and the [3H]flumazenil occupancy method, providing validation for the LC/MS methodology. With these data to support the assumption that the two techniques yield similar results, we further utilized the LC/MS method to obtain the receptor occupancy data shown in Fig. 2B for TPA023 and Cmpd 44. The LC/MS method demonstrated dose-dependent occupancy in the FC for these compounds.

Effects of GABA_A compounds on EEG and operant performance. Four animals were administered three repeated doses of vehicle via the subcutaneous route. Figure 3A shows the mean EEG spectral power data (left) and behavioral performance data in the concurrent operant task (right) obtained from these experiments. During administration of three successive vehicle doses over ~1.5 h of elapsed time, EEG spectral power in each of the five analyzed bands remained stable relative to the preinjection control as confirmed by statistical evaluation. Likewise, animals’ performance as measured by either response accuracy or latency in the auditory operant paradigm did not change significantly over the course of these vehicle dosing manipulations. Thus by these measures, rats sustained a stable EEG spectral structure and a uniform behavioral state during the ~2-h dosing regimen. This identical regimen was adhered to for all of drug administration experiments described subsequently, aside from evaluation of Cmpd 43 and Cmpd 44. In these two cases, rats were administered only a single dose on a given experimental day and subsequent activity evaluated continuously for 1.5 h. However, to compare these compounds to the others, a postdosing time envelope of EEG and behavioral changes identical to that in the multidosing experiments was analyzed.

**Fig. 3.** Dose-related effects of vehicle, lorazepam and zolpidem dosing on spectral EEG bands and concurrent behavioral performance parameters in the auditory operant task. Sulphobutylether-β-cyclodextrin vehicle (A), lorazepam (B) or zolpidem (C) were administered sc at escalating doses (lorazepam: 0.1, 0.3, 1.0 mg/kg; zolpidem: 0.1, 0.3, and 2.0 mg/kg), as EEG was collected continuously and animals performed the operant task. Left: mean (±SE, n = 4 animals; 3–6 replicates per mean) effect on spectral power in each indicated EEG band normalized as a percentage of predose level. Right: corresponding percentage of effects normalized to predose baseline on mean (±SE) accuracy and response latency obtained under the dosing condition. Three successive administrations of vehicle did not significantly affect any of the EEG bands or behavioral performance parameters. In contrast, lorazepam and zolpidem both elicited dose-dependent significant elevations in the δ- and β-EEG bands. Lorazepam also significantly increased γ and significantly decreased θ-band power. Administration of both lorazepam and zolpidem at the two higher doses also significantly impaired operant behavioral performance, as indicated by both the accuracy and response latency parameters (*P < 0.05; **P < 0.01; ***P < 0.001).
Administration of escalating lorazepam doses (0.1, 0.3, 1.0 mg/kg sc) in contrast to vehicle elicited robust and progressive dose-dependent changes in EEG spectral power and behavioral operant performance (Fig. 3B). The 0.1 mg/kg lorazepam dose did not produce measurable effects, but the 0.3 and 1.0 mg/kg doses markedly increased power in the \( \delta \) and \( \beta \)-EEG bands, increased \( \gamma \), and decreased \( \theta \)-power. These doses concurrently impaired behavioral performance in the operant task. Visual monitoring of behavior and the EEG revealed evidence, particularly at the 1.0 mg/kg dose, compatible with marked sedation-hypnosis, including generalized lack of locomotor activity, disengagement with the operant task, and, in the EEG, sporadic occurrence of large-amplitude slow-wave spindles, consistent with transition into slow-wave sleep. When this occurred, the operant chamber was opened briefly to arouse the animal and, as possible, maintain an awake state. These transient behavioral state changes, however, led to large standard errors in the mean normalized EEG in these animals, particularly in the lower frequency \( \delta \) and \( \alpha \)-bands.

Similar to lorazepam, administration of zolpidem also profoundly affected the EEG and operant behavioral performance (Fig. 3C). Zolpidem was administered in three escalating doses (0.1, 0.3, 2.0 mg/kg sc), and, like lorazepam, the two higher doses significantly elevated \( \delta \) and \( \beta \)-power in a dose-dependent manner. In contrast to lorazepam, however, this GABA\(_{A1}\)-selective drug did not modulate EEG power in either the \( \theta \)- or \( \gamma \)-bands over the tested dose range. Performance in the operant behavioral task was significantly disrupted with regard to both response accuracy (decreased) and response latency (increased) over the two higher doses. Similar to lorazepam, zolpidem also visibly impaired locomotor behavior and equilibrium, and animals often appeared to transition to a sleep state, particularly at the highest dose. Thus both lorazepam and zolpidem showed EEG signatures and overlapping overt behaviors in the tested dose ranges, consistent with sedation/hypnosis, as would be expected from their known psychopharmacological properties.

**Effects of GABA\(_{A2/3}\)-selective compounds on EEG and operant behavior.** We evaluated effects of increasing doses of TP003, TPA023 and each of the seven novel AstraZeneca compounds on EEG spectral power in behaving rats. Figure 4 shows the effects of three increasing doses of TPA023, AZD7325, and AZD6280 on EEG and behavior in the same format used in Fig. 3. The profile of all three compounds on EEG spectral bands was qualitatively similar. Mean EEG power in the lower frequency \( \delta \)-, \( \theta \)- or \( \alpha \)-bands was not affected, but significant increases were elicited in spectral power in both the \( \beta \)- and \( \gamma \)-bands by all three compounds. These effects were dose dependent with the 1.0 \( \mu \)mol/kg dose of each compound not producing a change, but the 10 and 30 \( \mu \)mol/kg doses of all three compounds, leading to significant and progressively greater effects. Importantly, despite the robust EEG \( \beta \)- and \( \gamma \)-band power increases, associated performance in the operant task was not notably impaired by any of the compounds across the tested dose ranges. Statistical evaluations of response accuracy and response latency did not indicate any significant changes relative to the predosing controls. Thus, in contrast to the nonselective GABA\(_{A1,2,3}\) drug, lorazepam, and to the GABA\(_{A1}\)-selective sedative-hypnotic, zolpidem, these GABA\(_{A2/3}\)-selective compounds produced clear elevation of EEG \( \beta \)- and \( \gamma \)-band oscillatory power without concurrent behavioral effects indicative of sedation.

Consistent with the findings for the three compounds shown in Fig. 3, TP003 and five additional AstraZeneca GABA\(_{A2/3}\)-selective compounds also induced dose-related increases in EEG spectral power confined to the \( \beta \)- and \( \gamma \)-bands, in conjunction with a lack of measurable impairment of operant performance. Figure 5 displays normalized EEG spectral power changes across the five frequency bands caused by the highest dose of zolpidem, lorazepam, and the full set of nine GABA\(_{A2/3}\)-selective compounds. This high dose of each of the compounds (excepting TP003 and Cmpd 30, which were not assessed for receptor occupancy) was on the saturating portion of in vivo receptor occupancy curve (see Table 2); thus it was assumed to produce near full occupancy of GABA\(_{A2/3}\) subunits. Figure 5 illustrates that all of these selective compounds at this high occupancy dose elicited significant spectral power increases restricted to the \( \beta \)- and \( \gamma \)-bands. Most also caused a modest decrease in \( \theta \)-band power, which did not achieve significance. Finally, none of the compounds at this near full occupancy dose significantly impaired performance accuracy or response latency parameters in the operant task that the animals performed throughout the EEG collection epochs (not shown). Thus this EEG signature in common to the nine distinct GABA\(_{A2/3}\)-selective compounds contrasted notably to that of zolpidem and lorazepam, both of which produced dramatic \( \delta \)-band elevations, as well as behavioral changes consistent with their marked sedative/hypnotic properties. This shared EEG signature, taken together with the structural diversity encompassed by this set of nine GABA\(_{A2/3}\)-selective compounds (Alhambra et al. 2011; Atack et al. 2006; Dias et al. 2005), supports a hypothesis that these EEG changes reflect an effect on ongoing oscillatory properties of cortical networks associated specifically with positive modulation of GABA\(_A\) receptors containing \( \alpha_{2/3} \)-subunits (see Discussion).

**Effects of lorazepam and GABA\(_{A2/3}\)-selective agents on behavioral performance in the conflict model of anxiety.** All compounds evaluated for EEG changes were also tested for effect on both punished and unpunished responding rates in the rat conflict model. Each compound dose was studied typically on 6–10 rats (range: 6–20 rats). Figure 6 summarizes the effects of increasing doses of zolpidem, lorazepam, TPA023, AZD628 and AZD7325 on punished and unpunished responding rates normalized as a percentage of control (predosing) rates. Zolpidem had small and variable effects on punished responding. As the dose was escalated, unpunished responding showed a trend to decrease, but this did not achieve significance at the highest administered dose (5 mg/kg).

Lorazepam, in contrast to zolpidem, statistically increased the rate of punished responding in a dose-dependent manner at 1 and 3 mg/kg po compared with vehicle control. A maximum increase to 1,121% of control rate of punished responding was achieved at 3 mg/kg. However, lorazepam concomitantly decreased unpunished responding at these same 1 and 3 mg/kg doses. Thus in this behavioral conflict model there was not a dose range of lorazepam that induced behavior associated with anxiolysis in the absence of sedative/hypnotic effects.

The GABA\(_{A2/3}\)-selective agents, TPA023, AZD6280 and AZD7325 showed a pattern of effects contrasting to both zolpidem and lorazepam. All produced significant dose-dependent elevations in punished responding rate. For all three
compounds, the threshold dose for a significant effect was 0.3 μmol/kg. Rates of punished responding further increased with escalating doses to a maximal normalized level ranging from 500–900% control at a 10–30 μmol/kg dose. Importantly, and in marked contrast to lorazepam, no significant effects on unpunished responding were indicated for any of the three compounds across the entire tested dose range. The remaining six GABA<sub>2/3</sub>-selective compounds were also evaluated similarly in this conflict model with an analogous result; all produced significant dose-dependent increases in punished response rate without affecting unpunished responding over a wide dose range (see Fig. 7), consistent with a property to elicit anxiolysis in the absence of overt sedation.

Coincidence of dose range affecting EEG and anxiolysis-related behavior for GABA<sub>2/3</sub> agents. That nine chemically divergent GABA<sub>2/3</sub>-subunit-selective compounds yielded consistent and dose-dependent effects on both the EEG γ-power and punished response rate in the conflict anxiolysis model raises the question of whether the dose ranges overlapped for these physiological and behavioral changes. Figure 7 addresses this question in a three-variable representation of dose vs. normalized γ-power elevation vs. normalized punished responding rate change achieved by the tested doses of lorazepam and each of the nine GABA<sub>2/3</sub>-selective compounds. Note that only the γ-spectral band was evaluated in this analysis, because only compounds that also raised the punished response rate increased γ-power. The conclusion emanating from this analysis is that the dose range for each of the compounds that led to progressive and increasingly significant levels of elevation in γ-power corresponded closely to the dose range that elicited significant elevation in punished responding rate. Although this relationship also held for lorazepam, the overlap common to all of the selective GABA<sub>2/3</sub> agents argues strongly that the EEG and anxiolytic changes are both dissociable from sedative side effects that are prevalent for lorazepam and other benzodiazepines. Thus, taken together, these results with the GABA<sub>2/3</sub>-selective compounds support a translational hypothesis preclinically that the γ-band...
power elevation induced by these agents provides a biomarker predictive of the anxiolytic dose range.

Relation between GABA$_{A_{2/3}}$-positive modulatory intrinsic activity and spectral EEG changes. Since GABA$_{A_{2/3}}$-Positive modulatory activity was quantified pharmacologically in the oocyte experiments, and dose-dependent EEG changes were also discreetly measurable, we finally addressed the question of a possible relationship for these two variables across the compound set. Hypothetically, a positive correlation would exist if the degree of modulation of GABA signaling specifically via GABA$_{A_{2/3}}$ subunits drove a dynamic range of spectral EEG change. However, to make valid comparisons across the compound set to appropriately address such a hypothesis, we needed to standardize the extent of EEG effect to a common level of GABA$_{A_{2/3}}$ receptor occupancy. Although the calculated receptor occupancy did not reach 100% for any of the compounds in the set (68–87%; Table 2), the dose-occupancy relation for each compound approached saturation at the highest tested dose (e.g., see Fig. 2). This observation justified an assumption that the highest administered doses were producing near full occupancy of GABA$_{A_{2/3}}$ subunits.

Based on this assumption, we examined by linear regression analysis the extent to which intrinsic modulatory efficacy measured for the compounds at a standardized dose of 1 μM in oocyte studies (Table 1) could account for the variance in normalized change in each defined EEG band at a saturating occupancy (30 μmol/kg) dose. Figure 8 shows the results of these regression analyses for GABA$_{A_{2/3}}$-selective modulatory efficacy vs. EEG $\beta$-, $\gamma$-, and $\delta$-band power elevation. Notably, $\gamma$-band EEG elevation induced by the compounds in vivo showed a significant positive correlation to modulatory efficacy for both GABA$_{A_{2/3}}$ (Fig. 8A) and GABA$_{A_{3}}$ (Fig. 8B) subunits measured in vitro. Although there was a trend for a similar correlation for $\beta$-band changes, the regressions did not achieve significance (Fig. 8, C and D).

Analogous linear regression analyses were also carried out for EEG changes in the $\delta$-, $\theta$-, and $\alpha$-spectral band vs. modulatory efficacy at GABA$_{A_{2/3}}$ and GABA$_{A_{3}}$ subunits (not shown). No apparent or significant correlations were found between EEG band changes in any of the six of these other analyses for this group of selective GABA$_{A_{2/3}}$ agents. In conclusion, these findings support the notion that the relative degree of EEG $\gamma$-band elevation induced by subtype-selective...
Nine chemically diverse compounds tested in this study all shared three features: 1) pharmacological efficacy to positively modulate GABAA channels containing \( \alpha_2 \)- and \( \alpha_3 \)-subunits over those containing \( \alpha_1 \)-, and in most cases \( \alpha_5 \)-subunits; 2) elicitation of dose-dependent elevation of EEG power in the \( \beta/\gamma \)-frequency bands; and 3) efficacy in absence of overt sedation in a conflict model of anxiety. We argue that the GABA\(_{\alpha_2/3}\) pharmacology (in contrast to some unknown off-target pharmacological feature) shared by the nine compounds likely is responsible for the uniform EEG and anxiolytic effects. Additional convincing support that the EEG spectral signature was driven by GABA\(_{\alpha_2/3}\) modulation derives from the finding that \( \gamma \)-EEG power elevation in vivo showed a significant positive correlation to the degree of modulatory efficacy measured in vitro across the set of compounds (Fig. 8).

GABA\(_{\alpha_2/3}\)-induced EEG changes partially overlap the benzodiazepine signature. \( \beta \)-Band power elevation is well established for benzodiazepines at behaviorally relevant doses both preclinically and clinically. Similarly, \( \gamma \)-band elevation in the 30- to 50-Hz range, although not as widely established in the literature, has been demonstrated with diazepam in rodent EEG (Krijzer et al. 1993; van Lier et al. 2004), and was readily detected here for lorazepam (Figs. 3, 5, and 7) over the same dose range affecting \( \beta \)-band activity. Likely, \( \gamma \)-band effects have not been as widely documented primarily due to the fact that EEG evaluation typically is constrained to a maximal \( \beta \)-band frequency of 30 Hz, particularly in clinical studies (e.g., Saleut et al. 2006). That both the GABA\(_{\alpha_2/3}\)-subtype-selective agent and nonselective benzodiazepine, lorazepam, produce \( \beta \)- and \( \gamma \)-band EEG power increases (Fig. 5) is most prudently explained by a conclusion that GABA\(_{\alpha_2/3}\) receptor modulation drives these effects. These findings also are consistent with those of Nickolls et al. (2011), showing that L-838,417, a GABA\(_A\)-positive allosteric modulator with \( \alpha_2\alpha_3 \)- and \( \alpha_2\alpha_5 \)-subunit selectivity induced a dose-dependent EEG \( \beta \)-band power increase in awake rats (they did not extend analysis to the higher frequency \( \gamma \)-band).

The compounds used in this study could not discriminate effects elicited by GABA\(_{\alpha_2}\) vs. GABA\(_{\alpha_3}\) subunit activation. These two pharmacological features tracked one another closely over a wide range of medicinal chemistry that generated the compounds included in this study (see Alhambra et al. 2011), as well as other drug discovery efforts (Atack 2009). However, evidence linking higher frequency EEG changes specifically to GABA\(_{\alpha_2/3}\) subunit modulation derives from the earlier studies of Kopp et al. (2004) that demonstrated selective amelioration of EEG \( \beta \)-band increases to diazepam in mice engineered with point-mutated diazepam-insensitive GABA\(_{\alpha_2}\) subunits (that study did not evaluate \( \gamma \)-band EEG power). Conversely, mice engineered with diazepam-insensitive GABA\(_{\alpha_3}\) subunits showed little effect vs. wild-type animals on the spectral EEG signature elicited by diazepam (Kopp et al. 2003), supporting the selective involvement of GABA\(_{\alpha_2}\) subunits in modulating higher frequency EEG effects.

The nonselective benzodiazepine, lorazepam, and the GABA\(_{\alpha_1}\)-selective drug, zolpidem, also produced EEG effects only partially overlapping the GABA\(_{\alpha_2/3}\)-selective agents. Most notably, both of these drugs produced robust \( \delta \)-band elevation relative to the baseline, indicating that this may be conferred either directly by GABA\(_{\alpha_1}\) signaling or secondarily to the behavioral state change produced by these compounds. In addition, lorazepam significantly decreased \( \theta \)-power. Although a similar trend was observed for the selective compounds, the

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### Table 2. In vivo receptor occupancy values for peripherally
dosed GABA\(_{\alpha_2/3}\)-subunit-selective positive allosteric modulators

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>Mean Maximum Receptor Occupancy in FC, %</th>
<th>Mean ED(_{50}) ( \mu \text{mol/kg} )</th>
<th>95% CI</th>
<th>Mean ED(_{80}) ( \mu \text{mol/kg} )</th>
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<td>4–6</td>
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<tr>
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<td>4.8</td>
<td>5–11</td>
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<tr>
<td>TPA-023( \dagger )</td>
<td>96</td>
<td>2.0</td>
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Mean Maximum Receptor Occupancy in FC denotes for each compound the number of maximal percent receptor occupancy in frontal cortex, as indicated by a sigmoidal curve fit with variable slope iteratively fit to the dose-occupancy data. Compounds are arranged top to bottom in the same order as in Table 1. Mean ED\(_{50}\) denotes for each compound calculated oral effective dose producing 50% of maximal occupancy (ED\(_{50}\)) in frontal cortex; 95% confidence interval (CI) for ED\(_{50}\) is shown. Mean ED\(_{80}\) denotes for each compound calculated oral effective dose producing 80% of maximal occupancy (ED\(_{80}\)) occupancy in frontal cortex. \( n \) denotes for each compound the number of animals from which mean occupancy data were obtained. \( \dagger \)Occupancy data shown for these compounds was derived with liquid chromatography-mass spectrometry method; all others were obtained using displacement of radioligand, \( [3H] \)flumazenil.
Possibly, this could be related to GABA-A/H9251 2,3 or GABA-A/H9251 5 signaling, but may require intrinsic efficacy higher than that associated with the subunit-selective agents tested here.

Interestingly, zolpidem overlapped with the GABA-A/H9251 2/3-subtype-selective agents in elevating - but not -power band. This observation indicates that effects of GABA-A modulators on - and -frequency bands as defined in our study are dissociable in rodent. The observation is also consistent with -power band increases being confined to positive modulatory at GABA-A/H9251 2 (and possibly GABA-A/H9251 3) channels. In as much as the effect of zolpidem can be considered GABA-A/H9251 1 selective, this observation indicates that effects of GABA-A modulators on - and -frequency bands as defined in our study are dissociable in rodent. The observation is also consistent with -power band increases being confined to positive modulatory at GABA-A/H9251 2 (and possibly GABA-A/H9251 3) channels. In as much as the effect of zolpidem can be considered GABA-A/H9251 1 selective, ...
this also suggests that interaction with both GABA_{A1} and GABA_{A2/3}-subunit-containing channels contributes to the cortical \( \beta \)-oscillatory power increase elicited by benzodiazepines (Fig. 5), although these data do not clarify whether a single brain network is involved in generating both effects. Although we cannot rule out a possibility that the exposure levels of zolpidem achieved in the present study elicited nonselective effect on GABA_{A2/3} channels, this would be difficult to reconcile with the differentiation of EEG changes, particularly related to the \( \gamma \)-frequency band.

**Revisiting the benzodiazepine pharmacological dissociation hypothesis.** The dose-dependent EEG \( \beta/\gamma \)-band elevation produced by GABA_{A2/3}-selective agents in absence of sedation necessitates a reexamination of the pharmacological dissociation concept between EEG and behaviorally induced effects of benzodiazepines discussed earlier (Coenen and van Luijtelaar 1991). Findings here clearly demonstrate \( \beta/\gamma \)-EEG power band elevation in response to GABA_{A2/3}-positive allosteric pharmacological modulation in the absence of evidence for sedation, as indicated by the lack of significant effects on response accuracy and latency in the operant task performed by the animals concurrently with the EEG collection. These findings present new understanding disfavoring the dissociation hypothesis of benzodiazepines. Rather, they support an alternative hypothesis that the \( \beta/\gamma \)-elevation in EEG power induced by GABA_{A2/3}-subunit modulation represents a direct pharmacological effect on brain networks, rather than a compensatory response secondarily to sedation.

**Involvement of cortical perisomatic interneurons in GABA_{A2/3}-mediated \( \gamma \)-oscillations.** Parvalbumin (PV) and cholecystokinin expressing GABAergic interneurons synapse widely on GABA_{A1} and GABA_{A2/3}-containing channels, respectively, on the perisomatic region of cortical pyramidal cells. This circuitry is believed integral to generating cortical \( \gamma \)-oscillations (reviewed by Freund and Katona 2007). More specifically, PV basket cells function in syncytial networks through divergent chemical and electrical synapses to synchronize firing in large populations of pyramidal cells via perisomatic recurrent inhibition. This circuitry has been hypothesized to function as a hardwired precise “clockwork” supporting \( \gamma \)-frequency oscillations via synaptic output onto GABA_{A1} containing channels (Freund 2003). Cholecystokinin-containing basket cells form an overlapping syncytial network that, in contrast, is believed through integration of feed-forward afferent input from subcortical inputs relevant to motivation and mood to fine tune oscillations produced by the PV/pyramidal network via postsynaptic GABA_{A2} containing channels (Freund 2003; Freund and Katona 2007). Nonetheless the exact contributions of these or other interneuron subsets to spontaneous or behaviorally modulated \( \gamma \)-oscillations has not been unequivocally determined.

Elegant studies employing optogenetic tools to alternatively activate or inhibit firing in the PV interneuron subset in cortical regions have provided functional evidence for a crucial role of these interneurons in generating \( \gamma \)-oscillations in vivo. Rhythmic optogenetic activation of PV interneurons was shown sufficient to drive \( \gamma \)-field potential oscillations in the somatosensory cortex (Cardin et al. 2009). Conversely, optogenetic inhibition of PV interneurons in FC suppressed \( \gamma \)-power evoked by coactivation of pyramidal cells (Sohal et al. 2009). These findings do not align clearly with our pharmacologically derived observation that selective GABA_{A1}-Positive modulation associated with zolpidem failed to affect spontaneous \( \gamma \)-power. However, Hines et al. (2013) recently engineered a viral probe that selectively disrupts the perisomatic clustering of GABA_{A2} subunits in FC. Following this treatment, they observed a profound suppression of spontaneous cortical EEG spectral power restricted to the \( \gamma \)-band with frequency cutoffs
nearly identical to those defined in the present study. Our findings are thus consistent with a hypothesis that positive modulation of the perisomatic GABAergic input at GABA_A receptors is involved at least partially in eliciting the spontaneous γ-band power increase common to the GABA_α_2/3-selective compounds tested here. More direct support for this hypothesis could come from future studies employing techniques such as selective optogenetic control of specific interneuron subsets in combination with GABA_α_2/3-selective agents such as those used in the present study.

Value of GABA_α_2/3-β/γ-EEG signature as a translational pharmacodynamic biomarker. Pragmatically, the EEG changes elicited by benzodiazepines in animals and humans represent a robust, noninvasive pharmacodynamic biomarker for positive modulation of GABA_A receptors, continuous in both magnitude and time. This is supported previously by elegant pharmacokinetic/pharmacodynamic analysis showing that the degree of rodent EEG β-band elevation for the nonselective GABA_A modulators, midazolam, bretazenil and RO 19-4603 is related closely to their differential intrinsic efficacy to enhance GABA signaling (Mandema et al. 1992). By extension, EEG effects produced by GABA_α_2/3-subtype-selective agents also qualify as a readily obtained pharmacodynamic biomarker related to modulation of oscillatory effects in brain circuits where these subunits are expressed. That the changes are related specifically to GABA_α_2/3 activation is supported by the significant positive correlation between in vitro pharmacological efficacy and degree to which these compounds enhanced γ-band EEG spectral power (Fig. 8). Although significant, the modest strength of these correlations could be related to our inability to deduce precise levels of receptor occupancy (compound exposure) during the time the EEG changes were being measured. Likewise, additional unexplained variance in the EEG could have been contributed by other subtle environmental or internal variables in these awake behaving animals.

Interestingly, the dose-dependent β/γ-EEG changes were elicited at the high end of the receptor occupancy range, typically in a dose range predicted to produce ED_80 to full saturating occupancy (compare Fig. 2 and Table 2 to Figs. 3 and 5). However, this was also coincident with the dose range conferring anxiolytic efficacy, as indicated by the conflict model of behavioral anxiety used here (Fig. 7). A similar occupancy-efficacy relationship was noted by Nickells et al. (2011) in their evaluation of TPA023 and L-838,417 in chronic pain models. This apparent offset between dynamic ranges of receptor occupancy and the EEG changes putatively signaling GABAergic functional modulation may result from the reduced maximal efficacy of these compounds relative to benzodiazepines. Such a relationship should be considered in designing translational clinical studies where a positron emission tomography ligand is used as a biomarker to inform dose range of GABA_α_2/3-subtype-selective agents in development. Using the β/γ-EEG band changes characterized here potentially have high value as an orthogonal means to gain confi-
dence that the dose range chosen achieved pharmacological effect on the brain networks targeted for efficacy.

ACKNOWLEDGMENTS

We thank the following individuals for contribution to the studies: Chad Elmore for purification of [3H]lumazenil; Geraldine Hill for validation of [3H]lumazenil binding in the rat brain; Teng Peng, Denis Hehman, Amy Hehman, and India Lynne Nevers for technical assistance; Barbara Pierce for formulation of compounds; and Drs. Chi Ming Lee and Tim Piser for critical reading of the manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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