EEG-β/γ spectral power elevation in rat: a translatable biomarker elicited by GABA_Aα2/3-positive allosteric modulators at nonsedating anxiolytic doses

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1Department of Neuroscience Biology, AstraZeneca Pharmaceuticals, Wilmington, Delaware; 2Department of Disposition, Metabolism and Pharmacokinetics, AstraZeneca Pharmaceuticals, Wilmington, Delaware; and 3Department of Chemistry, AstraZeneca Pharmaceuticals, Wilmington, Delaware

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Christian EP, Snyder DH, Song W, Gurley DA, Smolka J, Maier DL, Ding M, Ghahrengahi F, Liu XF, Chopra M, Ribadeneira M, Chapdelaine MJ, Dudley A, Arriza JL, Maciag C, Quirk MC, Doherty JJ. EEG-β/γ spectral power elevation in rat: a translatable biomarker elicited by GABA_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_A1, GABA_A2, and GABA_A5 subunits, modulate cortical network oscillations, as reflected by a complex signature in the EEG power spectrum. Recent drug discovery efforts have developed GABA_A2,3-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_A2,3-mediated vs. GABA_A1 or GABA_A5 currents in voltage clamped oocytes transfected with those GABA_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 anxiolysis without sedation. EEG γ-band power increases showed a significant positive correlation to in vitro GABA_A2,3 modulation intrinsic activity across the compound set, further supporting a hypothesis that this EEG signature was linked specifically to pharmacological modulation of GABA_A2,3 signaling. These findings encourage further evaluation of this EEG signature as a noninvasive clinical translational biomarker that could ultimately facilitate development of GABA_A2,3 subtype-selective drugs for anxiety and potentially other indications.

electroencephalography; benzodiazepine; GABA_A subtypes; pharmacokinetic; pharmacodynamic modeling; GABAergic interneurons via divergent innervation of pentameric GABA_A receptors on pyramidal neurons are fundamental to synchronizing cortical neuronal network oscillations that are reflected in EEG rhythms (Bartos et al. 2007; Fries et al. 2007). These rhythms recorded clinically from scalp, or from the cortical surface in animal models, are evaluated as quantitative 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 nonselectively at GABA_A1, GABA_A2,3, and GABA_A5 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 (Saleut et al. 2006; Gilles and Luthringer 2007). β-Band elevation has been proposed as a validated quantitative biomarker for GABA_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_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_A1- restricted positive modulators, such as zolpidem (Hoehns and Perry 1993), which act as selective sedative/hypnotics, but not anxiolytics. Conversely, as subtype-selective GABA_A2,3-poss-
itive 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 effects 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\textsubscript{A}\textsubscript{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\textsubscript{A}\textsubscript{2/3} subunit-selective modulators has been deduced indirectly by loss of function studies in genetically engineered mice in which the GABA\textsubscript{A}\textsubscript{3} (Kopp et al. 2003) or GABA\textsubscript{A}\textsubscript{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\textsubscript{A}\textsubscript{2/3.5}-subtype-selective positive modulator, L-838,417. They found that L-838,417, but not TPA023, elicits a dose-dependent increase in EEG b-band power. This raised the possibility that these EEG changes could represent a useful real-time biomarker if pharmacological specificity could be associated more conclusively with GABA\textsubscript{A}\textsubscript{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\textsubscript{A}\textsubscript{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\textsubscript{A}\textsubscript{1-3} subtype-selective modulator (Dias et al. 2005), and seven novel, subtype-selective GABA\textsubscript{A}\textsubscript{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\textsubscript{A}\textsubscript{1-3} subtypes) and zolpidem (selective positive modulation via GABA\textsubscript{A} subtypes) were also incorporated in the study as reference comparators.

Our results revealed that the GABA\textsubscript{A}\textsubscript{2/3} selective compounds all shared a dose-dependent EEG spectral signature comprised of \(\beta/\gamma\)-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\textsubscript{A}\textsubscript{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% \(\text{dH}_2\text{O}\) at 2 mg/ml stock concentration for the behavioral conflict studies, or 20% (wt/vol) sulphobutylether-\(\beta\)-cyclohexadecyl (SBCED; 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% SBCED (pH: 2.5) at 30 \(\mu\text{mol/ml}\) initial concentration and then further diluted in SBCED to the indicated doses. Seven novel putative GABA\textsubscript{A}\textsubscript{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 Cmpd 13 and 40 in Alhambra et al. (2011).

**Evaluation of GABA\textsubscript{A} \(\alpha_{1-5}\)-subtype-selective positive modulatory activity by two-electrode voltage clamp.** Recordings were made from *Xenopus laevis* oocytes injected with cRNA of GABA\textsubscript{A} subunits, leading to expression of GABA\textsubscript{A} \(\alpha_{1-2,3,5}\) receptors. These methods are described in detail by Alhambra et al. (2011). Briefly, two-electrode voltage clamp recording was carried out on eight oocytes simultaneously using OpusXpress 6000A (Molecular Devices, Sunnyvale, CA). An EC\textsubscript{10} concentration of GABA was applied to each oocyte, before and after preincubation for 100 s with each concentration of a GABA\textsubscript{A} modulatory compound. Positive modulation was normalized as a percentage of GABA current in the presence vs. absence of the modulator, as follows:

\[
\text{% potentiation} = \left[ \frac{(\text{GABA current amplitude with modulator})}{(\text{GABA current amplitude})} - 1 \right] \times 100
\]

The positive modulation produced by compounds was also normalized as a percentage of the modulation produced by a supramaximal (1 \(\mu\text{M}\)) diazepam concentration, to compare maximal positive modulatory efficacies against this reference drug. Concentration-response curves of these normalized values were plotted and fitted iteratively with a dose-response sigmoidal fitting equation with no imposed fitting constraints using Prism version 4.03 (GraphPad Software, San Diego, CA). The mean positive modulatory percent increases of the GABA EC\textsubscript{10} current produced by a 1 \(\mu\text{M}\) concentration each compound at channels composed of the four GABA\textsubscript{A} subtypes were compared statistically using one-way ANOVA, followed by Bonferroni multiple comparisons, to evaluate the significance of purported subtype selectivity.

**Animal subjects: in vivo studies.** Adult male rats, either Sprague-Dawley or Long Evans strains (Charles River, Wilmington, MA) as specified below were used in experiments. Rats tested with EEG recording and in the behavioral conflict model were singly housed, and those used in receptor occupancy studies were group housed. Rats were subjected to a 12:12 light-dark cycle (lights on 6 AM). Unless otherwise specified, animals were provided chow and water, ad libitum. All procedures performed on animals were approved by the AstraZeneca Institutional Animal Care and Use Committee in accordance with regulations established by Association for Assessment and Accreditation of Laboratory Animal Care.

**In vivo receptor occupancy studies.** Long Evans rats (150–170 g) were used for all studies. \(^{[3]}\text{H}\)flumazenil (Ro 15–1788; Perkin-Elmer) was employed as the radioligand. Radiochemical purity was monitored by HPLC prior to use to assure \(>97\%\) purity. The receptor occupancy protocol was modified from Goeders and Kuhar (1985),
Benavides et al. (1992), and Maier et al. (2009). Briefly, 1–2 days prior to the experiment, rats were instrumented surgically with an intraventricular jugular cannula. On the day of the experiment, vehicle (20% SBEC; volume 1 ml/kg po), the reference drug, diazepam (50 mg/kg ip), or test compound was administered to animals 30 min prior to the radioligand, [3H]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 nonselective 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:

\[
\% RO = 100 \times \left( \frac{\text{SB}_{\text{saline}} - \text{SB}_{\text{test compound}}}{\text{SB}_{\text{saline}}} \right)
\]

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 ED₅₀ and ED₉₀ 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 CH₃CN/H₂O (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 for the sample analysis. Multiple LC/MS experiments were performed to detect and isolate the fragment of the target ion. Peak area was used for measurements. Known concentrations of each compound were analyzed to generate a standard curve (\(r² > 0.99\)). Flumazenil in drug-treated samples was referenced against vehicle samples 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 electrospray 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 O₂). Body temperature was maintained at 37°C with a homeothermic blanket. Rats were surgically instrumented for skull surface EEG recording using standard stereotaxic techniques. Six stainless steel screws were placed in the skull to anchor the implant. Three screws also served as cortical surface electrodes for EEG acquisition. Electrode screw coordinates were as follows: 1) centrofrontal screw: (Bregma): anterior-posterior (A-P): +2.5 mm, L (left): 1.0 mm; 2) temporoparietal screw: A-P: −4.5 mm; L (left): 5.5 mm; 3) occipital reference screw: A-P: −10 mm, L: 0 mm. Screws were connected to a nanostrip connector (Omnetics, Minneapolis, MN) that was insulated and fixed to the skull with acrylic dental cement.

For recording, the electrode connector on the animal was mated to a headstage preamplifier (×20 gain; HST/16V-020; Plexon, Dallas, TX) attached to a flexible tether and commutator, allowing unimpeded movement of the animal in an operant chamber (11 in. length × 8.25 in. width × 13 in. height, metal grid of bars comprising floor; Med Associates, St. Alans, VT). Data were acquired via a Neuralynx (Bozeman, MT) Cheetah 24 channel system. Continuous EEG data were filtered at 1-Hz high pass, 325-Hz low pass, digitized at 32 kHz 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 receptacle containing an infrared photocell beam and detector was mounted 1.12 in. 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 photocell 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 compound testing protocol consisted first of acclimatizing 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 reintitated for a 30-min postdrug 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 two 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 Phr-
macological EEG Group Guidelines (see Versa et al. 1995), as follows: δ, 1–5.5 Hz; θ, 5.5–8.5 Hz; α, 8.5–12.5 Hz; β, 12.5–30 Hz; γ, 30–50 Hz without further subbanding the α- and β-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 $t_{\text{max}}$ 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 pre-dosing 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.

**Rate 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 ≥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$_{\text{A}2/3}$ subunits. The GABA$_{\text{A}2/3}$ subtype-selective modulator, TPA023 (Atack et al. 2006), and the putative GABA$_{\text{A}5}$ 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$_{\text{A}2/3}$ subtype selectivity for TPA023, for the putative GABA$_{\text{A}5}$ 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$_{\text{A}1}$, GABA$_{\text{A}2}$, GABA$_{\text{A}3}$ and GABA$_{\text{A}5}$ receptors, each heterologously reconstituted with GABA$_{\text{A}5}$ and GABA$_{\text{A}2/3}$ 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$_{\text{A}5}$ 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$_{\text{A}5}$ or GABA$_{\text{A}3}$ 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$_{\text{A}1}$ or GABA$_{\text{A}5}$ 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$_{\text{A}5}$- and GABA$_{\text{A}3}$-containing receptors, the GABA currents induced by all three compounds produced notably less maximal potentiation at these subtypes (~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 GABAA 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 GABAA$_{\alpha_2/3}$-containing channels vs. GABAA$_{\alpha_1}$-containing channels, as confirmed statistically. In addition, all of the compounds except Cmpd 30 and 43 showed significant functional selectivity for GABAA$_{\alpha_2/3}$ over GABAA$_{\alpha_5}$-containing channels. Additionally, Cmpd 30 and 43 and TPA023 were noted to show significant GABAA$_{\alpha_2}$ over GABAA$_{\alpha_3}$ Selectivity. Finally, the nine compounds spanned a continuum in the degree to which a 1 $\mu$M concentration increased GABA EC$_{10}$ current through GABAA$_{\alpha_2}$ or GABAA$_{\alpha_3}$ 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 GABAA 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 GABAA 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.
Table 1. Normalized potentiation of GABA-mediated currents produced by \( \text{GABA}_{\alpha2/3} \)-selective study compounds in oocytes expressing various \( \text{GABA}_{\alpha{\alpha}} \) subunits

<table>
<thead>
<tr>
<th>Subunit</th>
<th>( \alpha_1 )</th>
<th>( \alpha_2 )</th>
<th>( \alpha_3 )</th>
<th>( \alpha_5 )</th>
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<tr>
<td>AZD6280</td>
<td>14 ± 4 (6)***</td>
<td>91 ± 7 (11)</td>
<td>141 ± 12 (11)</td>
<td>16 ± 5 (4)***</td>
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<td>%DZ</td>
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<td>36 ± 4</td>
<td>34 ± 3</td>
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<td>AZD7325</td>
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<td>50 ± 3 (19)</td>
<td>47 ± 3 (10)</td>
<td>21 ± 3 (9)***</td>
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<td>19 ± 1</td>
<td>17 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Cmpd 29</td>
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<td>72 ± 5 (8)</td>
<td>92 ± 5 (11)</td>
<td>29 ± 3 (5)***</td>
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<td>25 ± 1</td>
<td>19 ± 1</td>
<td>11 ± 1</td>
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<td>Cmpd 30</td>
<td>12 ± 3 (14)*</td>
<td>31 ± 6 (10)</td>
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<td>19 ± 3</td>
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<tr>
<td>Cmpd 43</td>
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<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>57 ± 3 (7)</td>
<td>14 ± 10 (4)</td>
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<td>15 ± 2</td>
<td>17 ± 1</td>
<td>6 ± 2</td>
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%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_{\alpha1} and GABA_{\alpha5} currents that are significantly smaller than GABA_{\alpha2} current are noted (ANOVA, Bonferroni multiple comparisons; *P < 0.05, **P < 0.01, ***P < 0.001). GABA_{\alpha1} and GABA_{\alpha5} currents did not significantly differ for listed compounds, with the exception that GABA_{\alpha2} significantly exceeded GABA_{\alpha1} in case of AZD6280 (P < 0.01), compound (Cmpd) 30 (P < 0.05), Cmpd 43 (P < 0.001), and TPA-023 (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_{\alpha2/3} 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, \([3H]fumazenil\) (solid fits) and comparatively for AZD7325 by unlabeled fumazenil, 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 fumazenil 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. \([3H]fumazenil\) (see DISCUSSION).
curve and ED$_{50}$ 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 [$^3$H]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-$eta$-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 $\delta$- and $\beta$-EEG bands. Lorazepam also significantly increased $\gamma$, and significantly decreased $\theta$-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 δ- and β-EEG bands, increased γ, and decreased θ-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 δ- and α-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 δ- and β-power in a dose-dependent manner. In contrast to lorazepam, however, this GABAA1-selective drug did not modulate EEG power in either the θ- or γ-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-A1-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 δ-, θ- or α-bands was not affected, but significant increases were elicited in spectral power in both the β- and γ-bands by all three compounds. These effects were dose dependent with the 1.0 μmol/kg dose of each compound not producing a change, but the 10 and 30 μmol/kg doses of all three compounds, leading to significant and progressively greater effects. Importantly, despite the robust EEG β- and γ-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 GABAA, drug, lorazepam, and to the GABAA1-selective sedative-hypnotic, zolpidem, these GABAA2/3-selective compounds produced clear elevation of EEG β- and γ-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 β- and γ-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 β- and γ-bands. Most also caused a modest decrease in θ-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 δ-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 δ2/3-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 anxiolyis-related behavior for GABA<sub>2/3</sub> agents. That nine chemically divergent GABA<sub>2/3</sub>-selective compounds yielded consistent and dose-dependent effects on both the EEG <i>γ</i>-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 <i>γ</i>-power elevation vs. normalized punished responding rate achieved by the tested doses of lorazepam and each of the nine GABA<sub>2/3</sub>-selective compounds. Note that only the <i>γ</i>-spectral band was evaluated in this analysis, because only compounds that also raised the punished response rate increased <i>γ</i>-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 <i>γ</i>-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 <i>γ</i>-band...
power elevation induced by these agents provides a biomarker predictive of the anxiolytic dose range.

Relation between GABA<sub>A<sub>2/3</sub><sup>-</sup>-positive modulatory intrinsic activity and spectral EEG changes. Since GABA<sub>A<sub>2/3</sub><sup>-</sup>-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<sub>A<sub>2/3</sub><sup>-</sup>-positive 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<sub>A<sub>2/3</sub><sup>-</sup>-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<sub>A<sub>2/3</sub><sup>-</sup>-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 dose. Figure 8 shows the results of these regression analyses for GABA<sub>A<sub>2/3</sub><sup>-</sup>-selective agents commonly produced a moderate (nonsignificant) trend for θ-power suppression and significant elevation of β- and γ-power. Zolpidem, the only compound without GABA<sub>A<sub>2/3</sub><sup>-</sup>-modulatory capacity, was unique in that it did not affect γ-band power (*P < 0.05; **P < 0.01). Inset is a schematic summary of these results, showing the EEG spectral band power changes that are either unique to, or shared by, compounds positively modulating channels containing GABA<sub>A<sub>1</sub><sup>-</sup>, GABA<sub>A<sub>2</sub><sup>-</sup>, GABA<sub>A<sub>3</sub><sup>-</sup>, and GABA<sub>A<sub>5</sub><sup>-</sup>-subunits.

Fig. 5. Comparative EEG for all study compounds normalized as a mean percentage of control (+ SE; all AstraZeneca compounds: n = 4 – 6 animals; n = 5 – 8 replicates per mean) on each EEG band at highest tested dose (zolpidem: 2 mg/kg sc; lorazepam: 1 mg/kg sc; TP003: 25 μmol/kg sc; all AstraZeneca novel compounds and TPA023: 30 μmol/kg po). Note that zolpidem and lorazepam distinguished from all the GABA<sub>A<sub>2/3</sub><sup>-</sup>-selective agents in that both elicited a large increase in δ-band power. Lorazepam also significantly depressed θ-power and raised the higher frequency β- and γ-band powers. Likewise, all of the GABA<sub>A<sub>2/3</sub><sup>-</sup>-selective agents commonly produced a moderate (nonsignificant) trend for θ-power suppression and significant elevation of β- and γ-power. Zolpidem, the only compound without GABA<sub>A<sub>2/3</sub><sup>-</sup>-modulatory capacity, was unique in that it did not affect γ-band power (*P < 0.05; **P < 0.01). Inset is a schematic summary of these results, showing the EEG spectral band power changes that are either unique to, or shared by, compounds positively modulating channels containing GABA<sub>A<sub>1</sub><sup>-</sup>, GABA<sub>A<sub>2</sub><sup>-</sup>, GABA<sub>A<sub>3</sub><sup>-</sup>, and GABA<sub>A<sub>5</sub><sup>-</sup>-subunits.

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Table 2. In vivo receptor occupancy values for peripherally
dosed GABA_{A\alpha2/3} subunit-selective positive allosteric modulators

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Mean Maximum Receptor Occupancy in FC denotes for each compound the mean 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_{max} denotes for each compound calculated oral effective dose producing 80% of maximal occupancy (ED_{max}) occupancy in frontal cortex. n denotes for each compound the number of animals from which mean occupancy data were obtained. Occupancy data shown for these compounds was derived with liquid chromatography-mass spectrometry method; all others were obtained using displacement of radioligand, [3H]flumazenil.

GABA_{A\alpha2/3} agents can be predicted significantly by their measured pharmacological intrinsic efficacy to positively modulate GABA signaling via allosteric modulation selectively at GABA_{\alpha2} or GABA_{\alpha3} subunits.

**DISCUSSION**

Multiple drug discovery programs have endeavored to develop GABA_{A\alpha2/3}-subunit-selective positive allosteric modulators based on a hypothesis that these agents could deliver efficacy in absence of sedative/hypnotic and cognitive side effect liabilities endemic to nonselective GABA_{A} drugs, such as the benzodiazepines. Diverse therapeutic indications have been proposed, including generalized anxiety most prominently, but also cognitive deficits in schizophrenia (Lewis et al. 2004), and chronic pain (Knabl et al. 2008, Nickolls et al. 2011), as well as other drug discovery efforts (Atack 2009). That both the GABA_{A\alpha2/3}-subtype-selective agent and nonselective benzodiazepine, lorazepam, produce β- and γ-band EEG power increases (Fig. 5) is most prudently explained by a conclusion that GABA_{A\alpha2/3} 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 α_{3},3− and α_{5}-subunit selectivity induced a dose-dependent EEG β-band power increase in awake rats (they did not extend analysis to the higher frequency γ-band).

The compounds used in this study could not discriminate effects elicited by GABA_{A\alpha2} vs. GABA_{A\alpha3}-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_{A\alpha2/3}-subunit modulation derives from the earlier studies of Kopp et al. (2004) that demonstrated selective amelioration of EEG β-band increases to diazepam in mice engineered with point-mutated diazepam-insensitive GABA_{A\alpha2} subunits (that study did not evaluate γ-band EEG power). Conversely, mice engineered with diazepam-insensitive GABA_{A\alpha3} 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_{A\alpha2} subunits in modulating higher frequency EEG effects.

The nonselective benzodiazepine, lorazepam, and the GABA_{A\alpha1}-selective drug, zolpidem, also produced EEG effects only partially overlapping the GABA_{A\alpha2/3}-selective agents. Most notably, both of these drugs produced robust δ-band elevation relative to the baseline, indicating that this may be conferred either directly by GABA_{A\alpha1} signaling or secondarily to the behavioral state change produced by these compounds. In addition, lorazepam significantly decreased θ-power. Although a similar trend was observed for the selective compounds, the

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**TRANSLATIONAL EEG SIGNATURE OF GABA<sub>AA2/3</sub> MODULATORS**

Fig. 6. Dose-related effects of GABA<sub>A</sub> reference drugs, zolpidem (A) and lorazepam (B), and study exemplar GABA<sub>AA2/3</sub>-subtype-selective compounds [TPA023 (C), AZD6280 (D), and AZD7325 (E)] on the mean (± SE; n = 6–20 animals per mean) rate of responding normalized as a percent of predosing control in the punished and unpunished components of the behavioral conflict model for anxiety (*P < 0.05; **P < 0.01). Zolpidem across the tested dose range had no significant effect on either component, but at higher doses showed a trend to depress unpunished response rate. In contrast, lorazepam and all of the GABA<sub>AA2/3</sub>-selective compounds elicited a robust dose-dependent increase in punished responding rate. These increases were significant across a wide dose range for all of the GABA<sub>AA2/3</sub> agents. Note also that lorazepam was unique in producing dose-dependent depression of the unpunished responding component, and this occurred in the same dose range that elevated the punished response rate. The GABA<sub>AA2/3</sub>-selective compounds had no effect on unpunished response rate over the full dose range tested. This pattern shown for the GABA<sub>AA2/3</sub>-selective exemplars was characteristic of all the other study compounds with GABA<sub>AA2/3</sub>-selective pharmacology (see Results).

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effect only was significant for one of the nine agents (Fig. 5). Possibly, this could be related to GABA<sub>A<sub>α2</sub>,<sub>3</sub></sub> or GABA<sub>A<sub>α5</sub></sub> signaling, but may require intrinsic efficacy higher than that associated with the subunit-selective agents tested here.

Interestingly, zolpidem overlapped with the GABA<sub>A<sub>α2</sub>,<sub>3</sub></sub>-subtype-selective agents in elevating β- but not γ-band power. This observation indicates that effects of GABA<sub>A</sub> modulators on β- and γ-frequency bands as defined in our study are dissociable in rodent. The observation is also consistent with γ-power band increases being confined to positive modulatory at GABA<sub>A<sub>α2</sub></sub> (and possibly GABA<sub>A<sub>α3</sub></sub>) channels. In as much as the effect of zolpidem can be considered GABA<sub>A<sub>α1</sub></sub> selective,
this also suggests that interaction with both GABA_A1- and GABA_A2/3-subunit-containing channels contributes to the cortical β-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 γ-frequency band.

Revisiting the benzodiazepine pharmacological dissociation hypothesis. The dose-dependent EEG β/γ-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 β/γ-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 β/γ-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 γ-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 γ-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 γ-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 γ-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 γ-oscillations in vivo. Rhythmic optogenetic activation of PV interneurons was shown sufficient to drive γ-field potential oscillations in the somatosensory cortex (Cardin et al. 2009). Conversely, optogenetic inhibition of PV interneurons in FC suppressed γ-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 γ-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 γ-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\textsubscript{A2} receptors is involved at least partially in eliciting the spontaneous \(\gamma\)-band power increase common to the GABA\textsubscript{A2/3}-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\textsubscript{A2/3}-selective agents such as those used in the present study.

**Value of GABA\textsubscript{A2/3} \(\beta/\gamma\)-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 effects produced by GABAA receptors in animals and humans represent a robust, noninvasive pharmacodynamic biomarker for positive effects produced by GABAA receptors. 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\textsubscript{A2/3}-subtype-selective agents in development. Using the \(\beta/\gamma\)-EEG band changes characterized here potentially have high value as an orthogonal means to gain confi-
dence that the dose range chosen achieves pharmacological effect on the brain networks targeted for efficacy.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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