Ionotropic GABA receptor antagonism-induced adverse outcome pathways for potential neurotoxicity biomarkers

Antagonism of ionotropic GABA receptors (iGABARs) can occur at three distinct types of receptor binding sites causing chemically induced epileptic seizures. Here we review three adverse outcome pathways, each characterized by a specific molecular initiating event where an antagonist competitively binds to active sites, negatively modulates allosteric sites or noncompetitively blocks ion channel on the iGABAR. This leads to decreased chloride conductance, followed by depolarization of affected neurons, epilepsy-related death and ultimately decreased population. Supporting evidence for causal linkages from the molecular to population levels is presented and differential sensitivity to iGABAR antagonists in different GABA receptors and organisms discussed. Adverse outcome pathways are poised to become important tools for linking mechanism-based biomarkers to regulated outcomes in next-generation risk assessment.

Keywords: adverse outcome pathway • antagonist • chloride channel • cross-species extrapolation • epileptic seizure • ionotropic γ -aminobutyric acid receptor • metabotropic GABA receptor • neurotransmission • neurotoxicity biomarker • risk assessment

An adverse outcome pathway (AOP) is a conceptual construct that portrays existing knowledge concerning the linkage between the point at which a chemical interacts with a biological molecule such as a receptor, or a molecular initiating event (MIE), to key events that are measurable events that are essential for the pathway to proceed to an adverse outcome of regulatory interest [1]. It describes how molecular perturbations cause measurable effects that propagate in a causal manner from the molecular level to the organ, individual or population level. The AOP concept was initially introduced to causally link *in vitro* measurements to regulated outcomes in order to support hazard assessment and screening [1]. The concept was later recognized as a useful approach for chemical classification in forming toxicologically meaningful categories [2], and more recently advocated as a pragmatic tool with multiple toxicological applications such as the establishment of quantitative struc-

ture–activity relationships, the development of novel *in vitro* toxicity screening tests and the elaboration of prioritization strategies [3]. A number of AOPs have been developed in a public database (see aopwiki.org for details and the updated AOP list), and some well-defined AOPs have been published, including androgen receptor agonism [4], estrogen receptor antagonism [1] and aromatase inhibition [5,6] leading to reproductive dysfunction, skin sensitization related to modification of skin protein by the hapten (a sensitizing agent) [7,8], and cholestatic liver injury caused by drug-mediated bile salt export pump inhibition [9]. Here, we propose three new AOPs related to neurotoxicity induced by ionotropic γ -aminobutyric acid receptor (iGABAR) antagonism: iGABAR inhibition by competitive antagonism leading to epileptic seizures, iGABAR inhibition by negative allosteric modulation leading to epileptic seizures, and iGABAR inhibition by noncompetitive channel blocking leading

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to epileptic seizures. iGABARs represent an important pharmacological and toxicological target due to their ubiquitous distribution in the central nervous system (CNS), structural diversity and critical roles in inhibitory synaptic neurotransmission. We start with a brief introduction of the iGABAR, describe the AOPs and supporting evidence, and then discuss their complexity, potential use as biomarkers and implications for risk assessment.

iGABAR – a brief review

γ -aminobutyric acid (GABA), which is synthesized primarily from glutamate by glutamate decarboxylase, is the most widely distributed inhibitory neurotransmitter in the CNS of both invertebrates and vertebrates [10–12]. In invertebrates (e.g., insects), GABA mediates fast chemical neurotransmission along with *L*-glutamate, an excitatory neurotransmitter at nerve-muscle junctions [13]. In vertebrates, GABA acts at inhibitory synapses in the brain by binding to specific transmembrane receptors in the plasma membrane of both pre- and postsynaptic neuronal processes [12,14]. This binding causes the opening of ion channels to allow the flow of either negatively charged chloride ions into the neuron or positively charged potassium ions out of the neuron [12]. This action results in a negative change in the transmembrane potential, usually causing hyperpolarization [15,13,12]. There are also cases where GABA acts as an excitatory neurotransmitter due to increased intracellular Cl^- concentration during development of the nervous system [16] or in certain cell populations [17,18]. In such cases, the electrochemical potential of Cl^- within the neuron is higher than that of the extracellular environment and the electrochemical gradient pushes Cl^- ions out of the cell resulting in depolarization of membrane potential [12].

GABA can interact with two classes of receptors: Cys-loop ligand-gated ionotropic receptor (iGABAR) and G protein-coupled metabotropic receptor (mGABAR) [10,11]. In vertebrates, the iGABAR includes two subclasses of fast-responding ion channels, GABA_A receptor (GABA_A -R) and GABA_C receptor (GABA_C -R), whereas the mGABAR, also called GABA_B receptor (GABA_B -R), mediates slow response to GABA. GABA_A -Rs were first identified pharmacologically as being activated by GABA and the selective agonist muscimol, blocked by bicuculline and picrotoxin and modulated by benzodiazepines, barbiturates and certain other CNS depressants [19,20]. GABA_C -Rs have been designated as a subtype of GABA_A -Rs by the International Union of Pharmacology because they possess high structural similarities with but distinct pharmacological properties from other subtypes of GABA_A -Rs, for example, insensitivity to the typi-

cal antagonist bicuculline and allosteric modulators of GABA_A -R channels such as benzodiazepines and barbiturates [10–12,21]. Invertebrate iGABARs do not readily fit the vertebrate $\text{GABA}_A/\text{GABA}_C$ receptor categories. The majority of insect iGABARs are distinguished from vertebrate GABA_A receptors by their insensitivity to bicuculline and differ from GABA_C -Rs in that they are subject to allosteric modulation, albeit weakly, by benzodiazepines and barbiturates [22,23].

As a heteropentamer, the iGABAR consists of five protein subunits arranged around a central pore that constitutes the ion channel through the membrane (Figure 1A) [22,24–26]. Five subunits belonging to nineteen different GABA_A -R genes have been identified in the human genome, including six α , three β , three γ , one δ , one ϵ , one θ , one π and three ρ subunits [27]. Each subunit consists of a long N-terminal extracellular hydrophilic region, followed by four transmembrane (TM) α -helices with a large intracellular loop between TM3 and TM4 and ends with a relatively short extracellular C-terminal domain, and TM2 forms the lining of the ion channel (Figure 1B & 1C) [24,11,26]. In the extracellular domain of the GABA_A -R, there are five pockets at the five subunit interfaces. The two pockets at the β/α interface form the two GABA binding sites, and the pocket at the α/γ interface forms the benzodiazepine binding site (Figure 1B). The GABA pocket is formed by ‘loops’ A, B and C of the β^+ (‘principal’) side and ‘loops’ D, E and F of the α^- (‘complementary’) side [28].

The high diversity of subunit genes, in combination with alternative splicing and editing [22], leads to an enormous variety and, consequently, functional and pharmacological diversity of iGABAR subtypes having different subunit composition [11]. It has been estimated that as many as 800 distinct iGABAR subtypes may exist in mammals [29], even though only 26 native subtypes have been identified with varying degrees of evidence [25,11]. The majority of GABA_A -Rs are composed of one $\gamma 2$, two α and two β subunits, with the most common subtype in the mammalian CNS being $\alpha 1\beta 2\gamma 2$ [30]. GABA_C -Rs consist exclusively of ρ ($\rho 1$ – $\rho 3$) subunits, which can assemble into either homooligomeric or pseudohomooligomeric (e.g., $\rho 1\rho 2$) receptors [10]. Many iGABAR genes have been predicted in insect genomes. At least three subunits (*RDL*: resistant to dieldrin; *GRD*: GABA receptor of *Drosophila* and *LCCH3*: ligand-gated chloride channel homolog 3) and more of their alternative splicing/editing products have been characterized in insects [22].

Localized at both synaptic (pre- and postsynaptic) and extrasynaptic sites, iGABARs are widely distributed in many regions of the brain and other neurons of CNS and PNS [22,11]. GABA_A -Rs alone are involved in a

Figure 1. Structure of ionotropic GABA receptors based on the consensus in multiple literature reviews [10,21,23–25,30]. Shown is a common subtype $\alpha 1\beta 2\gamma 2$ of GABA_A receptors found in the mammalian CNS. **(A)** Five subunits from three subunit subfamilies assemble to form a heteropentameric chloride permeable channel. **(B)** Stoichiometry and subunit arrangement of the GABA_A receptor. Also shown are the binding sites for GABA and BZ. **(C)** Receptor subunits consist of four hydrophobic transmembrane domains (TM1–4), where TM2 is believed to line the pore of the channel. The large extracellular N-terminus is the site for ligand binding as well as the site of action of various drugs. Each receptor subunit also contains a large intracellular domain between TM3 and TM4, which is the site for various protein–protein interactions as well as the site for post-translational modifications that modulate receptor activity.

BZ: Benzodiazepines; CNS: Central nervous system; TM: Transmembrane.

number of CNS diseases, including sleep disturbances, anxiety, premenstrual syndrome, alcoholism, muscle spasms, Alzheimer's disease, chronic pain, schizophrenia, bipolar affective disorders and epilepsy [31,24]. There is also a growing body of evidence indicating their existence in non-neuronal tissues such as immune cells (e.g., T cells and macrophages [32]), endocrine glands (e.g., human and rat adrenal glands [33,34], heart, liver, intestine and reproductive organs (e.g., oviduct, uterus, ovary and testis of mammals and fathead minnow gonad) [35,34]. The main neurological functions of iGABARs have been shown to modulate anxiety, the excitability of the brain, muscle tonus, vigilance, learning and memory [36,37,12]. Recent reviews on both vertebrate and invertebrate iGABARs have covered the definition, pharmacology, localization, function, subunit composition, structure, abundance and stoichiometry of GABA_A-R, GABA_C-R and insect iGABAR (see [11,22,24–26,31]).

Description of AOPs & supporting evidence

The three AOPs for iGABAR antagonism leading to epileptic seizures, iGABAR inhibition by competitive antagonism leading to epileptic seizures, iGABAR inhibition by negative allosteric modulation leading to epileptic seizures and iGABAR inhibition by noncompetitive channel blocking leading to epileptic seizures, **Figure 2**, are each characterized by a specific MIE where a chemical antagonist binds to an active or an allosteric site on the iGABAR. The three distinct MIEs all result

in decreased Cl[−] conductance through the ligand-gated ion channel (LGIC), leading to effects at higher organizational levels shared by the three AOPs. The physiological outcome at the cellular level is typically decreased hyperpolarization (depolarization) of affected neurons, whereas the adverse effects at the organismal level are exemplified by the regulate outcome of epileptic seizures in humans [38–40]. The severity of symptoms is often dose- and duration-dependent, while the toxicological symptoms are associated with the type and location of affected iGABARs. Mortality can occur if the individual sustains a prolonged or pronounced convulsion or seizure. For ecological species, the regulated outcome is at the population level where the observed consequences are decreased growth rate and population decline. In the following, we provide supporting evidence for the MIEs and key events involved in these AOPs.

Classification of iGABAR antagonists

The iGABAR is a vulnerable target because a wide variety of substances from many different chemical classes, including both natural and man-made toxic agents such as insecticides and pharmacological and clinical drugs, have been found to interact with various active and allosteric sites in this LGIC complex [41–43,31,15]. Based largely on pharmacological, electrophysiological and biochemical evidence at the cellular level, at least 11 distinct ligand binding sites on GABA_A-Rs have been identified (e.g., those for GABA, bicuculline, barbiturates, benzodiazepines and

Figure 2. Adverse outcome pathways for ionotropic GABA receptor antagonism leading to epileptic seizures. The linear pathway of key events depicts a generalized framework for AOPs sharing three distinct molecular initiating events: iGABAR inhibition by competitive antagonism, iGABAR inhibition by negative allosteric modulation and iGABAR inhibition by noncompetitive channel blocking.

ACTH: Adrenocorticotrophin hormone; AOP: Adverse outcome pathway; CNS: Central nervous system; HPA: Hypothalamic–pituitary–adrenal; iGABAR: Ionotropic GABA receptor; LMBW: Longitudinal muscle of the body wall; NMJ: Neuromuscular junction; PNS: Peripheral nervous system; RDX: Research Demolition Explosive; sIPSC: Spontaneous inhibitory postsynaptic current.

picrotoxin), and many more are likely to emerge when the 3D structural information on various subtypes of GABA_A-Rs becomes available [31,44–46]. Active or orthosteric sites are the binding pockets of either agonists (e.g., GABA and muscimol) or antagonists (gabazine and bicuculline); whereas the allosteric binding sites are the targets of positive allosteric modulators (benzodiazepines and barbiturates), negative allosteric modulators (flumazenil and zinc) and noncompetitive channel blockers (picrotoxin and lindane) [47,31]. Allosteric ligands indirectly modulate the receptor activity (i.e., alter the response of the receptor to agonist) by binding to a site distinct from that of the orthosteric agonist binding site and inducing a conformational change within the receptor. For instance, the binding of the benzodiazepine class of tranquilizer drugs (benzodiazepine site) occurs at the γ +/ α - subunit interface or the α +/ β - subunit interface on the GABA_A-R [36,48]. Once bound, the benzodiazepine (as an allosteric agonist) locks the GABA_A-R into a conformation where the neurotransmitter GABA has much higher affinity for the GABA_A-R, increasing the frequency of opening of the associated chloride ion channel and hyperpolarizing the membrane. This potentiates the inhibitory effect of the available GABA leading to sedative and anxiolytic effects [48].

Here, we define an iGABAR antagonist as a chemical ligand that reduces Cl⁻ conductance through binding to

orthosteric or allosteric sites on the ion channel. According to this definition, iGABAR antagonists can be further divided into the following three categories [49,31]:

- Competitive antagonists that bind to the active orthosteric receptor site but do not activate it. These antagonists compete with GABA for binding, thereby inhibiting its action. Representative examples are bicuculline and gabazine which bind to GABA_A-Rs [50–52];
- Negative allosteric modulators (e.g., flumazenil and zinc) that bind to an allosteric site on the receptor complex and affect it in a negative manner, decreasing efficiency of the main active site and therefore an indirectly decreasing Cl⁻ conductance;
- Noncompetitive channel blockers, such as picrotoxin, fipronil, lindane [53] and cyclonite (commonly known as RDX, Research Department Explosive) [54], noncompetitively bind at or near the central pore of the receptor complex (e.g., the picrotoxin site) and directly block Cl⁻ conductance through the ion channel.

Evidence supporting the AOPs: from molecular to population levels

For each of the above three categories of iGABAR antagonism, we provide a summary of supporting evi-

dence for representative chemical ligands. However, it is not our intention to make an exhaustively complete list of chemicals belonging to each category, which is beyond the scope of this work. The mechanisms of iGABAR antagonism have been extensively studied and well characterized in the past five decades using a variety of technical approaches. The vast majority of molecular-level evidence is supported by experimental results obtained from numerous pharmacological, electrophysiological, biochemical and mutagenesis studies. Radioligand binding assays [55] have been widely used to evaluate the binding affinity of numerous compounds for GABARs; patch-clamp electrophysiology [56] allows high-resolution current recording of whole cells or excised cellular or tissue patches so that the physiological status of ion channels can be measured; a substituted-cysteine accessibility method (SCAM) [57] is often used to mutate pocket-forming amino acid residues in order to verify the ligand binding sites.

Molecular initiating events

Although a wealth of experimental data have pointed to which residues in the iGABAR are important for ligand binding and/or function, no consensus has been reached on how ligands bind and how ligands are oriented in the site. Recently, a number of computational studies on homology modeling and ligand docking [44,45,47,53,58–61] have been conducted to demonstrate ligand binding interactions between an antagonist and an iGABAR, and used the 3D x-ray structures of functional and structural homologs as templates in the absence of crystal structures of GABA_A-Rs. These homologs include a mouse nicotinic acetylcholine receptor (nAChR) $\alpha 1$ subunit [62], acetylcholine binding proteins [63] and prokaryotic pentameric LGICs from the bacteria *Erwinia chrysanthemii* [64,45] and *Gloeobacter violaceus* [65,66]. We focus here the molecular level evidence on recent computational findings that are supported by experimental data.

Competitive antagonists

Both bicuculline and gabazine have been found to dock to the binding domain of a high-quality $\alpha 1\beta 2\gamma 2$ GABA_A-R subtype model with $\beta 2$ E155 and $\alpha 1$ R66 acting as the main anchor points for the charged moieties in the ligands [44]. This is in good agreement with lines of experimental evidence showing that mutation of Tyr157 to Ser in the $\beta 2$ subunit of $\alpha 1\beta 2\gamma 2$ L rat GABA_A recombinant receptors resulted in bicuculline acting as a weak agonist [52]. For gabazine, a simultaneous H-bond to $\beta 2$ Y157 backbone carbonyl has been identified, and the carboxylate group formed a bidentate ionic interaction with $\alpha 1$ R119 [44]. The ring

systems in both ligands occupy the lower part of the pocket, an orientation consistent with SCAM data [67] showing that gabazine blocks modification of residues further down in the pocket than observed for GABA. The SCAM data also clearly suggest that the $\alpha 1$ D62- $\alpha 1$ S68 region is involved in forming the ligand binding site, and that the specific interactions of gabazine and the local structural movements associated with these interactions are mediated by different amino acid residues. Computational and experimental results suggest that the binding of an antagonist like gabazine, which is larger than GABA, prevents complete closure of the pocket thereby preventing channel gating.

Negative allosteric modulators

Richter *et al.* [61] evaluated several thousand docking positions of flumazenil and its close structural analogs by using multiple homology models and an unbiased docking protocol, overcame the low sequence identity (<20%) among the structural templates to construct 37 homology models of the GABA_A-R. Their results indicate that flumazenil can be best accommodated by the geometries and interactions in common binding mode I, which is also supported by a large variety of structural, computational and experimental evidence. For instance, the positions of common binding mode I $\alpha 1$ Tyr159 and $\alpha 1$ Tyr209, the key residues defining its L1 pocket, are notably similar to those of homologous residues of the ligand-bound complexes of both acetylcholine binding protein and nAChR.

Zinc ions are concentrated in the CNS and inhibit GABA_A receptor function, which are pivotal mediators of inhibitory synaptic neurotransmission. Hoise *et al.* [68] used molecular modeling to identify three discrete sites that mediate Zn²⁺ inhibition. One is located within the ion channel, and the other two are on the external amino (N)-terminal face of the receptor at the interfaces between α and β subunits. These three postulated types of Zn²⁺ binding site, formed by H267 and E270, and by E137, H141 and E182, act in a concerted, allosteric fashion to inhibit receptor function.

Noncompetitive channel blockers

Fipronil, lindane, α -endosulfan and picrotoxinin are important GABAergic insecticides. Despite large structural diversity, it has been postulated that they fit a single binding site in the chloride channel lumen lined by five TM2 segments. This hypothesis was examined with the $\beta 3$ homopentamer by mutagenesis, pore structure studies, ligand binding and molecular modeling [53]. Results suggest that all four chemicals fit the 2' to 9' pore region forming hydrogen bonds with the T6' hydroxyl and hydrophobic interactions with

A2', T6' and L9' alkyl substituents, thereby blocking the channel.

Key events at the cellular/tissue level

The key events at the cellular/tissue level are common to all three AOPs and are characterized by a dose-dependent inhibition of membrane currents in iGABAR-containing cells, especially neuronal cells [69].

Competitive antagonists

Bicuculline has been shown to competitively antagonized the firing action of GABA on cat [70] and mouse spinal cord, mouse cortical neurons in cell culture [51] and crayfish stretch receptor neurones [71]. Demuro *et al.* [72] demonstrated that membrane currents elicited by GABA in *Xenopus oocytes*, where three types of GABA_A receptors are expressed, were reversibly antagonized by bicuculline in a concentration-dependent manner. Behrens *et al.* [73] further showed that bicuculline and gabazine generated high frequency ripples on stimulus-induced sharp wave-ripple complexes in adult rat hippocampus *in vitro*, which has also been observed in epileptic humans and rats, indicated a loss of functional inhibition.

Negative allosteric modulators

Flumazenil abolishes the diazepam (a GABA agonist) or flurazepam (a benzodiazepine agonist) action upon the GABA_A-R channel in rat hippocampal neurons [16,74]. It acts by binding CNS benzodiazepine receptors and competitively blocking benzodiazepine activation of inhibitory GABAergic synapses [75,76]. It was also reported that the contents of the $\alpha 1$ and $\alpha 6$ receptor subunit mRNAs were significantly increased in the flumazenil-treated neuronal culture as compared with the dimethyl sulfoxide vehicle-treated control group, whereas there were no significant differences in the absolute amounts of the $\beta 2$, $\beta 3$, $\gamma 2S$, $\gamma 2L$ and δ receptor subunit mRNAs [77]. Results of double-immunolabeling experiments suggested that after chronic flumazenil treatment, receptor subunit assemblies containing the $\alpha 1/\gamma 2$ and $\alpha 6/\delta$ subunits were replaced by a receptor assembly containing the $\alpha 1/\delta$ subunit. Thus, flumazenil may also trigger a change in the expression and assembly of the subunits of the GABA_A receptor [77].

Amentoflavone has been shown to be a negative modulator of GABA-induced current at $\alpha 1\beta 2\gamma 2L$ and $\alpha 1\beta 1\gamma 2$ GABA_A receptors expressed in *Xenopus laevis* oocytes using a two-electrode voltage-clamp technique [78,79]. This action appears to be independent of the flumazenil-sensitive benzodiazepine modulatory sites on the GABA_A receptors.

Banerjee *et al.* [80] reported that Zn^{2+} dose-dependently inhibited muscimol (a GABA agonist)-stimulated

uptake of ^{36}Cl in rat cortical and cerebellar membranes. Kume *et al.* [81] studied autoradiographically the effect of Zn^{2+} on [3H]TBOB (t-[3H]butylbicycloorthobenzoate) binding to GABA_A receptors in rat brain, and revealed that Zn^{2+} inhibited [3H]TBOB binding in a dose-dependent manner in various brain regions with the greatest inhibition observed in cortical laminae I–III, most areas of hippocampus, striatum, septum and cerebellar cortex. Bicuculline and picrotoxin showed additive effects on [3H]TBOB binding, supporting the notion that Zn^{2+} acts at a site distinct from the picrotoxin and bicuculline sites on the GABA_A-R [81].

Noncompetitive channel blockers

Long-term exposure of primary cerebellar granule cell cultures to 3 μM dieldrin reduced the GABA_A receptor function to 55% of control, as measured by the GABA-induced ^{36}Cl uptake [82]. Juarez *et al.* observed that picrotoxin exerted concentration-dependent and reversible inhibition of GABA-induced membrane currents in primary cultured neurons obtained from the guinea-pig small intestine [83]. Williams *et al.* [54] reported that RDX bound exclusively to the picrotoxin convulsant site of the GABA_A ionophore. Whole-cell *in vitro* recordings in the rat basolateral amygdala showed that RDX reduces the frequency and amplitude of GABA_A-R-mediated spontaneous inhibitory postsynaptic currents and the amplitude of GABA-evoked postsynaptic currents. In extracellular field recordings from the basolateral amygdala, RDX-induced prolonged, seizure-like neuronal discharges. These pieces of cellular level evidence support that binding to the GABA_A receptor convulsant site is the primary mechanism of seizure induction by RDX and that the key event of reduction of GABAergic inhibitory transmission in the amygdala is involved in the generation of RDX-induced seizures [54].

Key events at the organ/nervous system level

The vast majority of iGABAR antagonists are convulsant agents primarily acting on the GABAergic neurotransmission system [50] and generally antagonizing GABA inhibition. Since the three iGABAR antagonism-induced neurotoxicity AOPs converge at key events at the cellular level, we will not differentiate between them at the organ and higher organizational levels. Here we use bicuculline, a competitive GABA_A receptor antagonist, as a representative example to discuss key events on CNS, PNS and neuromuscular junctions where iGABARs ubiquitously exist.

Bicuculline-induced epileptic seizures can lead to brain lesions in rats where up to 56% of the cortical neurons displayed type 1 injuries (progressive condensation of both karyo- and cytoplasm), and 9% showed

type 2 injuries (status spongiosus) [84]. Although the exact pathogenetic mechanisms are not known, the structural changes observed in the rat cerebral cortex are considered to be caused by astrocytic and to a lesser extent intraneuronal edema related to the seizure activity. The edema was mostly recoverable whereas neuronal alterations remained when recovery was instituted after 2 h of status epilepticus [85]. During the initial phase (30 min) of bicuculline-induced seizures, regional impairments of protein synthesis were observed in rat forebrain and cerebellum/brainstem as well as in extraneural tissues such as liver, intestine and kidney [86,87].

Bicuculline-induced epileptic seizures can also initiate brain inflammation in glia of guinea pig brain and promote blood-brain barrier damage that is independent of either leukocytes or blood-borne inflammatory molecules [88]. In turn, brain inflammation contributes to the duration and recurrence of seizures. On the other hand, co-administration of bicuculline can partially mitigate the exacerbating injury (hypoxic-ischemic encephalopathy) across the examined brain regions (the cortex, striatum, hippocampus and thalamus) in immature rats caused by allopregnanolone, a potent positive modulator of GABA_A-Rs [89].

GABA_A-R antagonists have been shown to influence the hypothalamic–pituitary–adrenal (HPA) axis [90,91], a major integrated system that maintains body homeostasis. The paraventricular nucleus is the hypothalamic region that uses corticotrophin-releasing hormone to stimulate the pituitary [92]. Upon stimulation, the anterior pituitary releases adrenocorticotrophin hormone, which is transported in general circulation to the adrenal cortex of the adrenal glands [93]. The adrenal glands, the effector organ of the HPA axis, rapidly synthesize and release cortisol into the blood where it participates in the response to stress and maintenance of homeostasis [92]. Locatelli *et al.* [90] observed that GABA_A-R antagonists such as bicuculline stimulated adrenocorticotrophin hormone and cortisol secretion, thereby interfering with the functionality of the HPA axis. From a clinical standpoint, bicuculline reversed the attenuating effects of moderate controlled cortical impact on the stress-induced HPA response [91], hence restored neuroendocrine stress response by GABA_A-R antagonists after experimental traumatic brain injury.

Cardiac vagal preganglionic neurons (CVPNs) are found predominantly in the nucleus ambiguus (NA) as well as dorsal motor nucleus of the vagus and in the intermediate zone between these two nuclei [94]. Responsible for the tonic, reflex and respiratory modulation of heart rate (HR), CVPNs receive substantial inputs from ionotropic receptors [95]. Microinjection of bicuculline into the NA of the brainstem evokes a

profound decrease in HR, demonstrating that there is a large GABAergic input to CVPNs that plays a role in setting the tonic level of HR [96]. Bicuculline also affects CNS regulation of blood glucose homeostasis in rats [97] because GABA_A receptors play an inhibitory role in the regulation of hepatic glucose metabolism [98] and injection of bicuculline methiodide (1–10 nmol) into the third ventricle is found to produce hepatic venous hyperglycemia in a dose-dependent manner [97]. Furthermore, bicuculline increases the mean arterial pressure, reduces the serum nitrite production and hence attenuate hypotension (low blood pressure) in endotoxin-treated rats, suggesting that a GABAergic mechanism is involved in endotoxin intoxication-associated hypotension [99].

Devlin [100] reviewed pharmacological and physiological studies performed on various GABAR subtypes found at the echinoderm neuromuscular junction located between the radial nerve and longitudinal muscle of the body wall. The author found that some actions of GABA on echinoderm muscle include regulation of basal tone and spontaneous rhythmic contractions and modulation of cholinergic responses, and bicuculline antagonizes GABA actions and produces a paradoxical effect on contractility, stimulating large protracted contractions of the longitudinal muscle of the body wall.

Adverse outcomes at the organismal level

The overall balance between neuronal excitation and inhibition is vital to normal brain function. Too much inhibition, or too little excitation, can lead to coma, depression, low blood pressure, sedation or sleep; on the other hand, too much excitation, or too little inhibition, can result in a range of conditions including convulsions, anxiety, high blood pressure, restlessness and insomnia [31]. Either imbalance in the extreme can result in death. The exact symptoms depend on what regions of the brain are involved and exactly what nerve cells are out of balance [31]. It has been well established that blockage of the GABA-gated chloride channel reduces neuronal inhibition and induces epileptic or epileptiform encephalopathy, the regulated adverse outcome for vertebrates. This may further lead to CNS hyperexcitation, convulsions and death [38–40]. Here we use two well-documented antagonists, fipronil and RDX, to demonstrate the adverse outcome in vertebrates as a result of iGABAR antagonism.

Fipronil is a relatively new phenylpyrazole insecticide designed to selectively inhibit insect GABA receptors with low vertebrate toxicity. It is widely used to control a broad spectrum of pests such as cockroaches, ants, beetles, termites, rice weevils, mole crickets and fleas in agricultural and residential environment [101–

[104]. Studies have shown that fipronil, at concentrations higher than that used for pest control, fipronil can affect a wide range of nontarget species, including human, rats, mice, birds and fish [102,105,104]. Exposure to fipronil produces hyperexcitation at low doses and convulsion or tonic-clonic epileptic seizure and seizure-related death at high doses [102,105]. The amount of fipronil needed to cause acute neurotoxicity varies widely across different species: it is highly toxic to honeybees ($LD_{50} = 0.004 \mu\text{g}/\text{bee}$), moderately toxic to lizards (LD_{50} for *Acanthodactylus dumerili* Lacertidae = $30 \mu\text{g a. i.}/\text{g bw}$), and gallinaceous birds ($LD_{50} = 11.3 \text{ mg/kg}$ for Northern bobwhite quail), somewhat toxic to laboratory mammals (oral $LD_{50} = 97 \text{ mg/kg}$ for rats; oral $LD_{50} = 91 \text{ mg/kg}$ for mice), but shows low toxicity to waterfowl ($LD_{50} > 2150 \text{ mg/kg}$ for mallard duck) and reversible intoxication in humans [105,104]. The chronic neurotoxic effects of fipronil include increased incidence of aggression, irritability to touch, epileptic seizures and death [105].

RDX is an explosive compound widely used during World War II. Neurotoxicological effects such as seizures, convulsions, and tremors have been reported in many vertebrate species including rats, deer mice, dogs, monkeys lizards and birds orally exposed to RDX for acute, intermediate or chronic durations [106,54]. Neurological symptoms that have been observed in humans include disorientation, lethargy, muscle twitching and marked hyperirritability. At higher doses ($\geq 40 \text{ mg RDX/kg/day}$), hyperactivity, hyperirritability, hyperreactivity and increased fighting have been observed in rats [106]. Invertebrates such as earthworms display ataxia and paralysis as the predominant signs of RDX poisoning, and such neurotoxicity is often reversible if animals are allowed to recover without further exposure [107]. A recent meta-analysis of genomic data in five species (Sprague-Dawley rat, Northern bobwhite quail, fathead minnow, earthworm and coral) suggests a common and conserved mode of action of RDX on the GABAergic neurotransmission pathway throughout these phylogenetically disparate organisms [108].

Adverse outcomes at the population level

Regulated outcomes for ecological species are generally focused upon population level impacts with the concern as to whether or not they are sustainable as a result of chemical exposure. Only a few studies exist on the consequences of iGABAR antagonism at the population level that are limited to several widely used insecticides like fipronil and dieldrin. In general, the widespread use of insecticides not only decreases the population size of target species but also can cause a population decline of nontarget organisms. There are 49 reported field incidents where the use of fipronil in

rice seed treatment caused massive crawfish kills when the rice field tailwater was released into canals and used to irrigate fish ponds [109]. Aerial spraying of fipronil to control a plague of migratory locust (short-horned grasshopper) in Madagascar between 1997 and 2000 resulted in massive shrimp kills and 80–95% destruction of termite colonies, a key part of nutrient recycling and the food web, even 6 months postspray [110]. This triggered an even greater unanticipated consequence: disruption of the food chain due to an overall depletion of invertebrate prey (termites), and a subsequent decline of several insectivorous, termite-specialized vertebrate populations such as lizards and hedgehog tenrec [110,104].

There also exist long-term population impact studies for another insecticide dieldrin. It was reported that during 1963–1975, at least 29% of sparrowhawks *Accipiter nisus* found in an eastern British area with a high use rate of cyclodiene insecticides (aldrin, dieldrin and heptachlor) died directly from dieldrin poisoning, compared with only 6% in a more western area of low cyclodiene use [111,112]. Sibly *et al.* further estimated the quantitative effects of cyclodiene use on sparrowhawk populations in four different scenarios corresponding to (1) continued high cyclodiene use, (2) with and (3) without the inclusion of sublethal effects and density-dependence and (4) reduced cyclodiene use during subsequent recovery period 1976–82 [113]. Using a simplified life history model, they determined the rate of sparrowhawk population decline at 20, 60 and 12% per year in scenarios (1), (2) and (3), respectively, and a rate of 13% per year population growth in scenario (4). Based on field data, their analyses and predictions reasonably represented the realistic true impact of dieldrin and other cyclodienes on British sparrowhawk population [113].

Discussion

Differential sensitivity between vertebrates & invertebrates

Differential sensitivity in iGABAR antagonism can be viewed from two opposite standpoints. First, different chemicals possess differential potency on the same iGABAR in the same species. For instance, insecticides fipronil and dieldrin exhibit differential actions on GABA-gated chloride channels in American cockroach thoracic ganglia [114]. Fipronil inhibits GABA-evoked currents with an IC_{50} value of 28 nM , whereas dieldrin elicits a dual action potentiation with an EC_{50} value of 4 nM followed by inhibition with an IC_{50} value of 16 nM . Fipronil inhibition is partially reversible, whereas dieldrin inhibition is irreversible. While both fipronil and dieldrin act on the resting receptor at comparable rates, fipronil blocks the acti-

vated receptor 10-times faster than dieldrin [114]. In addition, although α -endosulfan, lindane and fipronil possess similar potency on the recombinant $\beta 3$ homooligomeric receptor (IC_{50} values of 0.5–2.4 nM) and the $\alpha 1\beta 3\gamma 2$ heterooligomeric subtype (IC_{50} values of 16–33 nM), they are highly selective (i.e., differentially potent) on the native brain receptor (IC_{50} values of 7.3, 306 and 2470 nM, respectively) [115], suggesting that differential potency is subtype-dependent.

Second and also more importantly, different species display differential sensitivity to the same chemical, leading to selective toxicity or species selectivity. Species selectivity and acquired resistance to neuroactive insecticides are attributable to a number of factors, including structural differences in binding subsites, receptor subunit interfaces and transmembrane regions [101,116]. For instance, fipronil exhibits higher potency with house fly $\beta 3$ GABAR than with human $\alpha 1\beta 2\gamma 2$ GABAR, likely due to the different subunit assemblies of these GABARs, because analyses of Surflex-docking suggest that the H-bond interaction of fipronil with Ala2 and Thr6 in the TM2 segment of GABARs plays a relatively important role in ligand selective binding [59]. Zhang *et al.* further studied the insensitivity of GABA_A-R competitive antagonists bicuculline and gabazine to the $\rho 1$ GABA_C receptor, and found that bicuculline insensitivity was mainly determined by Tyr106, Phe138 and Phe240 residues, whereas gabazine insensitivity was highly dependent on Tyr102, Tyr106 and Phe138 [117].

In addition to a higher efficacy on insect iGABARs, the specificity of fipronil on insects may also come from the facts that it can block the passage of chloride ions through both the iGABAR and the glutamate-gated chloride (GluCl) channels [118,114], and that GluCl channels do not exist in mammals [119,13]. The lower potency of its metabolite fipronil sulphone suggests that metabolic conversion serves as a detoxifying process in mammalian brain [120].

Animals may also exhibit differential sensitivity to the same chemical at different developmental stages. For instance, zinc (50–300 μ M) reversibly antagonized the GABA response in embryonic and young postnatal neurons of rat superior cervical ganglia, while neurons cultured from adult animals were far less sensitive and occasionally resistant to zinc blockade [121]. The differential blocking effect of zinc on cultured neurons of different ages also occurred in intact superior cervical ganglia tissue [121].

Effects of iGABAR antagonists on non-GABAergic receptors & non-neuronal targets

The ionotropic GABARs are members of the cyst-loop receptor family of LGICs. All members of this super

family have a signature cysteine loop, two cysteine residues separated by 13 other residues, in the amino-terminal domain of each subunit [49]. Besides iGABARs, this family also includes subfamilies of nAChRs, serotonin receptor type 3, glycine receptors, zinc-activated cation channel and invertebrate glutamate/serotonin-activated anionic channels [49,122,123]. Hence, it is not surprising that compounds blocking the chloride channels of iGABARs may also act on other members of the cyst-loop super family.

For instance, fipronil, which is developed to target insect GABA receptors, was recently found inhibiting a structurally related glycine receptor subtype in zebrafish, causing developmental neurotoxicity measured as notochord degeneration during the development of spinal locomotor pathways [124]. This represents an unanticipated and potentially novel mechanism for fipronil toxicity in vertebrates.

Another good example is the invertebrate GluCl channels that, like iGABARs, also play an important role in the inhibitory neurotransmission in the invertebrate nervous systems [38,125]. Many iGABAR antagonists including fipronil, dieldrin, hexachlorocyclohexane (γ -HCH) and 4'-ethynyl-4-n-propylbicycloorthobenzoate (EBOB) have been found blocking actions of both GABA- and glutamate-induced responses in American cockroach neurons [126,118,114]. The low selectivity of EBOB cautions the use of [³H] EBOB as a radioligand at high concentrations in binding assays to evaluate the pharmacology of GABA-gated chloride channels [126].

The distribution of iGABARs in non-neuronal cells means that iGABAR antagonists can elicit multiple toxicological effects, varying from behavioral to reproductive disorders. However, these effects are far less well characterized than neurotoxicity in the form of epileptic seizures with only a limited number of studies available. For instance, one study demonstrates that perinatal exposure to picrotoxin during the critical periods of male brain sexual differentiation has long-term effects on the reproductive physiology and causes decreased sexual behavior of male rats [127]. Another study shows that at doses causing neurotoxicity, endosulfan, a GABA_A-R antagonist, also elicits increased cancer, genotoxicity, endocrine disruption and developmental or reproductive toxicity in rodents [128]. RDX appears to be another good example because experimental data suggest that RDX affects hematological system (e.g., decreases in hemoglobin and erythrocyte levels), impairs liver functions (decreases in blood glucose, alanine aminotransferase, serum triglyceride and/or cholesterol levels and increases in serum cholesterol levels) and elicits carcinogenicity (hepatic adenoma and carcinoma) and reproductive toxicity

(increased incidence of spermatic granuloma in the rat prostate and decreased fecundity in invertebrates such as earthworms and enchytraeid potworms) [106,129,130].

Implications for risk assessment

The AOPs for iGABAR antagonism leading to epileptic seizures, along with an increasing number of other AOPs, offer a new resource for computational or predictive toxicology and systems biology which can be integrated with high-throughput and *in vitro* testing and used to assist in the identification of novel biomarkers within the risk assessment process [1,131,132,73]. In order to make this useful for mechanism-based risk assessment, particular attention must be paid to the complications brought by two issues discussed above.

Differential sensitivity & cross-species extrapolation

Cross-species extrapolation (CSE) is one of the most critical issues in regulatory toxicology and risk assessment because of the lack of toxicity data for many organisms potentially exposed to and affected by environmental contaminants [4]. On the other hand, a minimum of four species is often required to provide an acceptable level of uncertainty for predictive modeling [133]. Collecting toxicological data even for this minimum number of representative species is time-consuming and cost-prohibitive. Current practice in dealing with uncertainty for model predictions based on limited toxicological data of only two species is to increase the uncertainty factor by 10,000 [133], which largely reduces the toxicity threshold and results in excessive penalties and remediation costs. Therefore, an important obstacle that CSE faces is: how do chemical risk assessors apply the threshold obtained for a model species used in standardized toxicity testing to the species present at a contaminated site? The fundamental question underlying this obstacle is: how does toxicity vary among different species, given the fact that both evolutionary conservation and divergency exist in the toxicant-targeted molecular entities between phylogenetically disparate species [4,134]? Specifically, in the case of species sensitivity to iGABAR antagonism-induced neurotoxicity, fundamental research is needed to answer how much and why does receptor diversity affect species sensitivity? These fundamental questions have begun to be answered for some iGABAR antagonists through new computational and experimental approaches [101,53,59,103,67,68,126,83,60,36,44,45,117,114]. Answering these questions not only improves the accuracy of risk assessment by reducing the uncertainty in CSE, but can result in cost-saving from unnecessary remediation operations due to the application of an overconserved

approach currently based on the use of uncertainty factors.

Other toxic effects of iGABAR antagonists

The toxicity of iGABAR antagonists through MIEs other than GABAergic neurons adds another layer of complexity to the application of this AOP to regulatory toxicology and risk assessment because the adverse impacts at higher organizational levels (e.g., whole animal) may result from multiple MIEs. These other AOPs, in combination with receptor (target) sensitivity, determine the selectivity and efficacy of biologically active chemicals such as drugs and insecticides. A plausible approach to assessing environmental and health risks associated such chemicals is to generate as many AOPs, each with a distinct MIE, as there exist. Based on these distinct AOPs, it would be feasible to model and quantitatively predict the adverse outcome of a single chemical possessing multiple MIE or a mixture of chemicals. There is no doubt that the AOP community will focus on establishing a quantitative relationship between MIEs and risk assessment endpoints in the near future [2,132,8].

Conclusion & future perspective

The AOPs for iGABAR antagonism leading to epileptic seizures provide a generalized framework of toxicological pathways where antagonism of the iGABAR causes seizure-related mortality and adverse impacts on population growth via three distinct MIEs that block chloride channels on GABAergic neurons. Chemicals activating or interacting with these MIEs can be classified into three subcategories: competitive antagonist, negative allosteric modulator and noncompetitive channel blocker. Here we presented supporting evidence for key events observed across multiple biological organization levels including molecular, cellular, tissue/tissue, organ, whole organism and population levels. These AOPs comprehensively apply to a large variety of structurally distinct chemicals as well as a wide spectrum of diversified iGABARs in both vertebrates and invertebrates. When applying these AOPs to risk assessment, the diversity and differential sensitivity of iGABARs and the possible multiple MIEs an iGABAR antagonist may possess should be considered. Although there remain complicating and unsolved issues as discussed above, we anticipate that these AOPs will become an important tool in chemical classification and identification of novel biomarkers [2,117] and constitute as an essential part of mechanism-based next-generation risk assessment [1,131,132,8,3].

Disclaimer

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Army Corps of Engineers. Any mention of commercial products is for clarification and not intended as an endorsement.

Financial & competing interests disclosure

The authors (P Gong and EJ Perkins) would like to acknowledge the US Army Environmental Quality and Installations Basic Research Program and the "Rapid Hazard Risk Assess-

ment" Focus Area Research Project for financial support. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Background

- An adverse outcome pathway (AOP) represents a sequential chain of causally linked events composed of a molecular initiating event linked to essential key events and ultimately an adverse outcome of regulatory concern.
- As sensitive molecular species, ionotropic γ -aminobutyric acid receptors (iGABARs) may interact with a wide variety of substances from many different chemical classes, causing neurotoxic effects such as epileptic seizures.

Classification of iGABAR antagonists & AOP supporting evidence

- Three iGABAR antagonism-induced neurotoxicity AOPs are proposed, corresponding to three subcategories of iGABAR antagonists: competitive antagonists, negative allosteric modulators and noncompetitive channel blockers.
- For each of these AOPs, supporting evidence for molecular initiating events, key events and adverse outcomes is provided.

Implications for biomarker development & risk assessment

- Different chemicals possess differential potency on the same iGABAR in the same organism.
- Different organisms display differential sensitivity to the same chemical, leading to selective toxicity or cross-species toxicity variations.
- Cross-species extrapolation of AOP-based biomarkers and side effects of iGABAR antagonists are the two areas that warrant further research.

Conclusion

- AOP is a promising tool applicable to chemical classification, mechanism-based biomarker development and next-generation risk assessment.

References

- 1 Ankley GT, Bennett RS, Erickson RJ *et al.* Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.* 29(3), 730–741 (2010).
- 2 OECD. Report of the workshop on using mechanistic information in forming chemical categories. No. 138, 1–176 (2011).
- 3 Vinken M. The adverse outcome pathway concept: a pragmatic tool in toxicology. *Toxicology* 312, 158–165 (2013).
- 4 Lalone CA, Villeneuve DL, Cavallin JE *et al.* Cross-species sensitivity to a novel androgen receptor agonist of potential environmental concern, spironolactone. *Environ. Toxicol. Chem.* 32(11), 2528–2541 (2013).
- 5 Breen M, Villeneuve DL, Ankley GT *et al.* Developing predictive approaches to characterize adaptive responses of the reproductive endocrine axis to aromatase inhibition: II. Computational modeling. *Toxicol. Sci.* 133(2), 234–247 (2013).
- 6 Villeneuve DL, Breen M, Bencic DC *et al.* Developing predictive approaches to characterize adaptive responses of the reproductive endocrine axis to aromatase inhibition: I. Data generation in a small fish model. *Toxicol. Sci.* 133(2), 225–233 (2013).
- 7 Mackay C, Davies M, Summerfield V, Maxwell G. From pathways to people: applying the adverse outcome pathway (AOP) for skin sensitization to risk assessment. *ALTEX* 30(4), 473–486 (2013).
- 8 Maxwell G, Mackay C, Cubberley R *et al.* Applying the skin sensitisation adverse outcome pathway (AOP) to quantitative risk assessment. *Toxicol. In Vitro* 28(1), 8–12 (2014).
- 9 Vinken M, Landesmann B, Goumenou M *et al.* Development of an adverse outcome pathway from drug-mediated bile salt export pump inhibition to cholestatic liver injury. *Toxicol. Sci.* 136(1), 97–106 (2013).
- 10 Bormann J. The 'ABC' of GABA receptors. *Trends Pharmacol. Sci.* 21(1), 16–19 (2000).
- 11 Olsen RW, Sieghart W. GABA A receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology* 56(1), 141–148 (2009).
- 12 Watanabe M, Maemura K, Kanbara K, Tamayama T, Hayasaki H. GABA and GABA receptors in the central nervous system and other organs. *Int. Rev. Cytol.* 213, 1–47 (2002).
- 13 Raymond-Delpech V, Matsuda K, Sattelle BM, Rauh JJ, Sattelle DB. Ion channels: molecular targets of neuroactive insecticides. *Invert. Neurosci.* 5(3–4), 119–133 (2005).

- 14 Yamada K, Yu B, Gallagher JP. Different subtypes of GABAB receptors are present at pre- and postsynaptic sites within the rat dorsolateral septal nucleus. *J. Neurophysiol.* 81(6), 2875–2883 (1999).
- 15 Narahashi T, Ginsburg KS, Nagata K, Song JH, Tatebayashi H. Ion channels as targets for insecticides. *Neurotoxicology* 19(4–5), 581–590 (1998).
- 16 Taketo M, Yoshioka T. Developmental change of GABA(A) receptor-mediated current in rat hippocampus. *Neuroscience* 96(3), 507–514 (2000).
- 17 Cherubini E, Giaresia JL, Ben-Ari Y. GABA: an excitatory transmitter in early postnatal life. *Trends Neurosci.* 14(12), 515–519 (1991).
- 18 Lamsa K, Taira T. Use-dependent shift from inhibitory to excitatory GABAA receptor action in SP-O interneurons in the rat hippocampal CA3 area. *J. Neurophysiol.* 90(3), 1983–1995 (2003).
- 19 Macdonald RL, Olsen RW. GABAA receptor channels. *Annu. Rev. Neurosci.* 17, 569–602 (1994).
- 20 Sieghart W. Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol. Rev.* 47(2), 181–234 (1995).
- 21 Chebib M, Johnston GA. The 'ABC' of GABA receptors: a brief review. *Clin. Exp. Pharmacol. Physiol.* 26(11), 937–940 (1999).
- 22 Buckingham SD, Biggin PC, Sattelle BM, Brown LA, Sattelle DB. Insect GABA receptors: splicing, editing, and targeting by antiparasitics and insecticides. *Mol. Pharmacol.* 68(4), 942–951 (2005).
- 23 Hosie AM, Aronstein K, Sattelle DB, French-Constant RH. Molecular biology of insect neuronal GABA receptors. *Trends Neurosci.* 20(12), 578–583 (1997).
- 24 Michels G, Moss SJ. GABAA receptors: properties and trafficking. *Crit. Rev. Biochem. Mol. Biol.* 42(1), 3–14 (2007).
- 25 Olsen RW, Sieghart W. International union of pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol. Rev.* 60(3), 243–260 (2008).
- 26 Sarto-Jackson I, Sieghart W. Assembly of GABA(A) receptors (review). *Mol. Membr. Biol.* 25(4), 302–310 (2008).
- 27 Simon J, Wakimoto H, Fujita N, Lalande M, Barnard EA. Analysis of the set of GABA(A) receptor genes in the human genome. *J. Biol. Chem.* 279(40), 41422–41435 (2004).
- 28 Ernst M, Brauchart D, Boresch S, Sieghart W. Comparative modeling of GABA(A) receptors: limits, insights, future developments. *Neuroscience* 119(4), 933–943 (2003).
- 29 Barnard EA, Skolnick P, Olsen RW *et al.* International union of pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. *Pharmacol. Rev.* 50(2), 291–313 (1998).
- 30 Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* 101(4), 815–850 (2000).
- 31 Johnston GA. GABA(A) receptor channel pharmacology. *Curr. Pharm. Des* 11(15), 1867–1885 (2005).
- 32 Mendu SK, Bhandage A, Jin Z, Birnir B. Different subtypes of GABA-A receptors are expressed in human, mouse and rat T lymphocytes. *PLoS One* 7(8), e42959- (2012).
- 33 Kato K, Nakagawa C, Murabayashi H, Oomori Y. Expression and distribution of GABA and GABA -receptor in the rat adrenal gland. *J. Anat.* 224(2), 207–215 (2013).
- 34 Metzeler K, Agoston A, Gratzl M. An Intrinsic gamma-aminobutyric acid (GABA)ergic system in the adrenal cortex: findings from human and rat adrenal glands and the NCI-H295R cell line. *Endocrinology* 145(5), 2402–2411 (2004).
- 35 Biggs K, Seidel JS, Wilson A, Martyniuk CJ. gamma-Aminobutyric acid (GABA) receptor subunit and transporter expression in the gonad and liver of the fathead minnow (*Pimephales promelas*). *Comp Biochem. Physiol. A Mol. Integr. Physiol* 166(1), 119–127 (2013).
- 36 Ramerstorfer J, Furtmuller R, Sarto-Jackson I, Varagic Z, Sieghart W, Ernst M. The GABAA receptor alpha+beta-interface: a novel target for subtype selective drugs. *J. Neurosci.* 31(3), 870–877 (2011).
- 37 Soto PL, Ator NA, Rallapalli SK *et al.* Allosteric modulation of GAB receptor subtypes: effects on visual recognition and visuospatial working memory in Rhesus monkeys. *Neuropsychopharmacology* 38(11), 2315–2325 (2013).
- 38 Bloomquist JR. Chloride channels as tools for developing selective insecticides. *Arch. Insect Biochem. Physiol.* 54(4), 145–156 (2003).
- 39 De Deyn PP, Marescau B, Macdonald RL. Epilepsy and the GABA-hypothesis a brief review and some examples. *Acta Neurol. Belg.* 90(2), 65–81 (1990).
- 40 Werner FM, Covenas R. Classical neurotransmitters and neuropeptides involved in generalized epilepsy: a focus on antiepileptic drugs. *Curr. Med. Chem.* 18(32), 4933–4948 (2011).
- 41 Chebib M, Johnston GA. GABA-Activated ligand gated ion channels: medicinal chemistry and molecular biology. *J. Med. Chem.* 43(8), 1427–1447 (2000).
- 42 Jett DA. Chemical toxins that cause seizures. *Neurotoxicology* 33(6), 1473–1475 (2012).
- 43 Johnston GA, Hanrahan JR, Chebib M, Duke RK, Mewett KN. Modulation of ionotropic GABA receptors by natural products of plant origin. *Adv. Pharmacol.* 54, 285–316 (2006).
- 44 Sander T, Frolund B, Bruun AT, Ivanov I, McCammon JA, Balle T. New insights into the GABA(A) receptor structure and orthosteric ligand binding: receptor modeling guided by experimental data. *Proteins* 79(5), 1458–1477 (2011).
- 45 Spurny R, Billen B, Howard RJ *et al.* Multisite binding of a general anesthetic to the prokaryotic pentameric *Erwinia chrysanthemi* ligand-gated ion channel (ELIC). *J. Biol. Chem.* 288(12), 8355–8364 (2013).
- 46 Vijayan RS, Trivedi N, Roy SN *et al.* Modeling the closed and open state conformations of the GABA(A) ion channel – plausible structural insights for channel gating. *J. Chem. Inf. Model.* 52(11), 2958–2969 (2012).

- 47 Ernst M, Bruckner S, Boresch S, Sieghart W. Comparative models of GABAA receptor extracellular and transmembrane domains: important insights in pharmacology and function. *Mol. Pharmacol.* 68(5), 1291–1300 (2005).
- 48 Sigel E, Luscher BP. A closer look at the high affinity benzodiazepine binding site on GABAA receptors. *Curr. Top. Med. Chem.* 11(2), 241–246 (2011).
- 49 Chang Y, Huang Y, Whiteaker P. Mechanism of allosteric modulation of the Cys-loop receptors. *Pharmaceuticals* 3(8), 2592–2609 (2010).
- 50 Johnston GA. Advantages of an antagonist: bicuculline and other GABA antagonists. *Br. J. Pharmacol.* 169(2), 328–336 (2013).
- 51 Nowak LM, Young AB, Macdonald RL. GABA and bicuculline actions on mouse spinal cord and cortical neurons in cell culture. *Brain Res.* 244(1), 155–164 (1982).
- 52 Ueno S, Bracamontes J, Zorumski C, Weiss DS, Steinbach JH. Bicuculline and gabazine are allosteric inhibitors of channel opening of the GABAA receptor. *J. Neurosci.* 17(2), 625–634 (1997).
- 53 Chen L, Durkin KA, Casida JE. Structural model for gamma-aminobutyric acid receptor noncompetitive antagonist binding: widely diverse structures fit the same site. *Proc. Natl Acad. Sci. USA* 103(13), 5185–5190 (2006).
- 54 Williams LR, Ioniadou-Anderjaska V, Qashu F *et al.* RDX binds to the GABA(A) receptor-convulsant site and blocks GABA(A) receptor-mediated currents in the amygdala: a mechanism for RDX-induced seizures. *Environ. Health Perspect.* 119(3), 357–363 (2011).
- 55 Frey KA, Albin RL. Receptor binding techniques. *Curr. Protoc. Neurosci.* Chapter 1, Unit 1.4 (2001).
- 56 Bebarova M. Advances in patch clamp technique: towards higher quality and quantity. *Gen. Physiol. Biophys.* 31(2), 131–140 (2012).
- 57 Liapakis G, Simpson MM, Javitch JA. The substituted-cysteine accessibility method (SCAM) to elucidate membrane protein structure. *Curr. Protoc. Neurosci.* Chapter 4, Unit 4.15 (2001).
- 58 Campagna-Slater V, Weaver DF. Molecular modelling of the GABAA ion channel protein. *J. Mol. Graph. Model.* 25(5), 721–730 (2007).
- 59 Cheng J, Ju XL, Chen XY, Liu GY. Homology modeling of human alpha 1 beta 2 gamma 2 and house fly beta 3 GABA receptor channels and Surflex-docking of fipronil. *J. Mol. Model.* 15(9), 1145–1153 (2009).
- 60 Nury H, Van RC, Weng Y *et al.* X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel. *Nature* 469(7330), 428–431 (2011).
- 61 Richter L, de GC, Sieghart W *et al.* Diazepam-bound GABAA receptor models identify new benzodiazepine binding-site ligands. *Nat. Chem. Biol.* 8(5), 455–464 (2012).
- 62 Dellisanti CD, Yao Y, Stroud JC, Wang ZZ, Chen L. Crystal structure of the extracellular domain of nAChR alpha1 bound to alpha-bungarotoxin at 1.94 Å resolution. *Nat. Neurosci.* 10(8), 953–962 (2007).
- 63 Celie PH, van Rossum-Fikkert SE, van Dijk WJ, Brejc K, Smit AB, Sixma TK. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. *Neuron* 41(6), 907–914 (2004).
- 64 Hilf RJ, Dutzler R. X-ray structure of a prokaryotic pentameric ligand-gated ion channel. *Nature* 452(7185), 375–379 (2008).
- 65 Bocquet N, Nury H, Baaden M *et al.* X-ray structure of a pentameric ligand-gated ion channel in an apparently open conformation. *Nature* 457(7225), 111–114 (2009).
- 66 Hilf RJ, Dutzler R. Structure of a potentially open state of a proton-activated pentameric ligand-gated ion channel. *Nature* 457(7225), 115–118 (2009).
- 67 Holden JH, Czajkowski C. Different residues in the GABA(A) receptor alpha 1T60-alpha 1K70 region mediate GABA and SR-95531 actions. *J. Biol. Chem.* 277(21), 18785–18792 (2002).
- 68 Hosie AM, Dunne EL, Harvey RJ, Smart TG. Zinc-mediated inhibition of GABA(A) receptors: discrete binding sites underlie subtype specificity. *Nat. Neurosci.* 6(4), 362–369 (2003).
- 69 Dichter MA, Ayala GF. Cellular mechanisms of epilepsy: a status report. *Science* 237(4811), 157–164 (1987).
- 70 Curtis DR, Duggan AW, Felix D, Johnston GA. Bicuculline, an antagonist of GABA and synaptic inhibition in the spinal cord of the cat. *Brain Res.* 32(1), 69–96 (1971).
- 71 McLennan H. Bicuculline and inhibition of crayfish stretch receptor neurons. *Nature* 228(5272), 674–675 (1970).
- 72 Demuro A, Martinez-Torres A, Francesconi W, Miledi R. Antagonistic action of picrotoxin on human and rat GABA(A) receptors. *Br. J. Pharmacol.* 127(1), 57–64 (1999).
- 73 Behrens CJ, van den Boom LP, Heinemann U. Effects of the GABA(A) receptor antagonists bicuculline and gabazine on stimulus-induced sharp wave-ripple complexes in adult rat hippocampus *in vitro*. *Eur. J. Neurosci.* 25(7), 2170–2181 (2007).
- 74 Weiss M, Tikhonov D, Buldakova S. Effect of flumazenil on GABAA receptors in isolated rat hippocampal neurons. *Neurochem. Res.* 27(12), 1605–1612 (2002).
- 75 Hoffman EJ, Warren EW. Flumazenil: a benzodiazepine antagonist. *Clin. Pharm.* 12(9), 641–656 (1993).
- 76 Votey SR, Bosse GM, Bayer MJ, Hoffman JR. Flumazenil: a new benzodiazepine antagonist. *Ann. Emerg. Med.* 20(2), 181–188 (1991).
- 77 Zheng TM, Caruncho HJ, Zhu WJ *et al.* Chronic flumazenil alters GABA(A) receptor subunit mRNA expression, translation product assembly and channel function in neuronal cultures. *J. Pharmacol. Exp. Ther.* 277(1), 525–533 (1996).
- 78 Hanrahan JR, Chebib M, Davucheron NL, Hall BJ, Johnston GA. Semisynthetic preparation of amentoflavone: a negative modulator at GABA(A) receptors. *Bioorg. Med. Chem. Lett.* 13(14), 2281–2284 (2003).
- 79 Hansen RS, Paulsen I, Davies M. Determinants of amentoflavone interaction at the GABA(A) receptor. *Eur. J. Pharmacol.* 519(3), 199–207 (2005).
- 80 Banerjee PK, Olsen RW, Snead OC, III. Zinc inhibition of gamma-aminobutyric acid(A) receptor function is decreased in the cerebral cortex during pilocarpine-induced status epilepticus. *J. Pharmacol. Exp. Ther.* 291(1), 361–366 (1999).

- 81 Kume A, Sakurai SY, Albin RL. Zinc inhibition of t-[3H] butylbicycloorthobenzoate binding to the GABAA receptor complex. *J. Neurochem.* 62(2), 602–607 (1994).
- 82 Babot Z, Vilario MT, Sunol C. Long-term exposure to dieldrin reduces gamma-aminobutyric acid type A and N-methyl-D-aspartate receptor function in primary cultures of mouse cerebellar granule cells. *J. Neurosci. Res.* 85(16), 3687–3695 (2007).
- 83 Juarez EH, Ochoa-Cortes F, Miranda-Morales M, Espinosa-Luna R, Montano LM, Barajas-Lopez C. Selectivity of antagonists for the Cys-loop native receptors for ACh, 5-HT and GABA in guinea-pig myenteric neurons. *Auton. Autacoid. Pharmacol.* 34(1–2), 1–8 (2013).
- 84 Soderfeldt B, Kalimo H, Olsson Y, Siesjo B. Pathogenesis of brain lesions caused by experimental epilepsy. Light- and electron-microscopic changes in the rat cerebral cortex following bicuculline-induced status epilepticus. *Acta Neuropathol.* 54(3), 219–231 (1981).
- 85 Soderfeldt B, Kalimo H, Olsson Y, Siesjo BK. Bicuculline-induced epileptic brain injury. Transient and persistent cell changes in rat cerebral cortex in the early recovery period. *Acta Neuropathol.* 62(1–2), 87–95 (1983).
- 86 Kiessling M, Xie Y, Kleihues P. Regional impairment of protein synthesis in the rat brain during bicuculline-induced seizures. *Brain Res.* 296(1), 1–13 (1984).
- 87 Kiessling M, Kleihues P. Regional protein synthesis in the rat brain during bicuculline-induced epileptic seizures. *Acta Neuropathol.* 55(2), 157–162 (1981).
- 88 Librizzi L, Noe F, Vezzani A, de CM, Ravizza T. Seizure-induced brain-borne inflammation sustains seizure recurrence and blood-brain barrier damage. *Ann. Neurol.* 72(1), 82–90 (2012).
- 89 Tsuji M, Taguchi A, Ohshima M, Kasahara Y, Ikeda T. Progesterone and allopregnanolone exacerbate hypoxic-ischemic brain injury in immature rats. *Exp. Neurol.* 233(1), 214–220 (2012).
- 90 Locatelli V, Bresciani E, Tamiazzo L, Torsello A. Central nervous system-acting drugs influencing hypothalamic-pituitary-adrenal axis function. *Endocr. Dev.* 17, 108–120 (2010).
- 91 Taylor AN, Tio DL, Sutton RL. Restoration of neuroendocrine stress response by glucocorticoid receptor or GABA(A) receptor antagonists after experimental traumatic brain injury. *J. Neurotrauma* 30(14), 1250–1256 (2013).
- 92 Tsigos C, Chrousos GP. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J. Psychosom. Res.* 53(4), 865–871 (2002).
- 93 Engelmann M, Landgraf R, Wotjak CT. The hypothalamic-neurohypophyseal system regulates the hypothalamic-pituitary-adrenal axis under stress: an old concept revisited. *Front. Neuroendocrinol.* 25(3–4), 132–149 (2004).
- 94 Corbett EK, Batten TF, Kaye JC, Deuchars J, McWilliam PN. Labelling of rat vagal preganglionic neurones by carbocyanine dye DiI applied to the heart. *Neuroreport* 10(6), 1177–1181 (1999).
- 95 Hildreth CM, Goodchild AK. Role of ionotropic GABA, glutamate and glycine receptors in the tonic and reflex control of cardiac vagal outflow in the rat. *BMC Neurosci.* 11, 128 (2010).
- 96 DiMicco JA, Gale K, Hamilton B, Gillis RA. GABA receptor control of parasympathetic outflow to heart: characterization and brainstem localization. *Science* 204(4397), 1106–1109 (1979).
- 97 Nonogaki K, Iguchi A, Sakamoto N. Bicuculline methiodide influences the central nervous system to produce hyperglycemia in rats. *J. Neuroendocrinol.* 6(4), 443–446 (1994).
- 98 Nonogaki K, Iguchi A. Role of central neural mechanisms in the regulation of hepatic glucose metabolism. *Life Sci.* 60(11), 797–807 (1997).
- 99 Hsu DZ, Liu MY. Effect of GABA(A) receptor antagonist bicuculline methiodide on hypotension in endotoxin-intoxicated rats. *Toxicology* 200(2–3), 213–219 (2004).
- 100 Devlin CL. The pharmacology of gamma-aminobutyric acid and acetylcholine receptors at the echinoderm neuromuscular junction. *J. Exp. Biol.* 204(Pt 5), 887–896 (2001).
- 101 Casida JE, Durkin KA. Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. *Annu. Rev. Entomol.* 58, 99–117 (2013).
- 102 Gunasekara AS, Truong T, Goh KS, Spurlock F, Tjeerdema RS. Environmental fate and toxicology of fipronil. *J. Pestic. Sci.* 32(3), 189–199 (2007).
- 103 Hainzl D, Cole LM, Casida JE. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. *Chem. Res. Toxicol.* 11(12), 1529–1535 (1998).
- 104 Tingle CC, Rother JA, Dewhurst CF, Lauer S, King WJ. Fipronil: environmental fate, ecotoxicology, and human health concerns. *Rev. Environ. Contam. Toxicol.* 176, 1–66 (2003).
- 105 Jackson D, Cornell CB, Luukinen B, Buhl K, Stone D. Fipronil Technical Fact Sheet (2009).
- 106 Abadin H, Smith C, Ingerman L, Lladós FT, Barber LE, Plewak D, & Diamond GL. Toxicological Profile for RDX (2012).
- 107 Gong P, Inouye LS, Perkins EJ. Comparative neurotoxicity of two energetic compounds, hexanitrohexaazaisowurtzitan and hexahydro-1,3,5-trinitro-1,3,5-triazine, in the earthworm *Eisenia fetida*. *Environ. Toxicol. Chem.* 26(5), 954–959 (2007).
- 108 Garcia-Reyero N, Habib T, Pirooznia M *et al.* Conserved toxic responses across divergent phylogenetic lineages: a meta-analysis of the neurotoxic effects of RDX among multiple species using toxicogenomics. *Ecotoxicology* 20(3), 580–594 (2011).
- 109 US EPA (United States Environmental Protection Agency). Fipronil Summary Document Registration Review: initial docket. Docket Number: EPA-HQ-OPP-2011-0448. Case No. 7423 (2011).
- 110 Peveling R, McWilliam AN, Nagel P *et al.* Impact of locust control on harvester termites and endemic vertebrate predators in Madagascar. *J. Appl. Ecol.* 40, 729–741 (2003).
- 111 Walker CH, Newton I. Effects of cyclodiene insecticides on the sparrowhawk (*Accipiter nisus*) in Britain - a reappraisal of the evidence. *Ecotoxicology* 7, 185–189 (1998).

- 112 Walker CH, Newton I. Effects of cyclodiene insecticides on raptors in Britain - correction and updating of an earlier paper by Walker & Newton, *Ecotoxicology* 7, 175–189 (1998). *Ecotoxicology* 8, 423–427 (1999).
- 113 Sibly RM, Newton I, Walker CH. Effects of dieldrin on population growth rates of sparrowhawks 1963–1986. *J. Appl. Ecol.* 37, 540–546 (2000).
- 114 Zhao X, Salgado VL, Yeh JZ, Narahashi T. Differential actions of fipronil and dieldrin insecticides on GABA-gated chloride channels in cockroach neurons. *J. Pharmacol. Exp. Ther.* 306(3), 914–924 (2003).
- 115 Ratna GS, Kamita SG, Casida JE. Role of human GABA(A) receptor beta3 subunit in insecticide toxicity. *Toxicol. Appl. Pharmacol.* 172(3), 233–240 (2001).
- 116 Ozoe Y, Akamatsu M. Non-competitive GABA antagonists: probing the mechanisms of their selectivity for insect versus mammalian receptors. *Pest. Manag. Sci.* 57(10), 923–931 (2001).
- 117 Zhang J, Xue F, Chang Y. Structural determinants for antagonist pharmacology that distinguish the rho1 GABAC receptor from GABAA receptors. *Mol. Pharmacol.* 74(4), 941–951 (2008).
- 118 Ikeda T, Zhao X, Kono Y, Yeh JZ, Narahashi T. Fipronil modulation of glutamate-induced chloride currents in cockroach thoracic ganglion neurons. *Neurotoxicology* 24(6), 807–815 (2003).
- 119 Narahashi T, Zhao X, Ikeda T, Nagata K, Yeh JZ. Differential actions of insecticides on target sites: basis for selective toxicity. *Hum. Exp. Toxicol.* 26(4), 361–366 (2007).
- 120 Li P, Akk G. The insecticide fipronil and its metabolite fipronil sulphone inhibit the rat alpha1beta2gamma2L GABA(A) receptor. *Br. J. Pharmacol.* 155(5), 783–794 (2008).
- 121 Smart TG, Constanti A. Differential effect of zinc on the vertebrate GABAA-receptor complex. *Br. J. Pharmacol.* 99(4), 643–654 (1990).
- 122 Connolly CN, Wafford KA. The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. *Biochem. Soc. Trans.* 32(Pt3), 529–534 (2004).
- 123 Raymond V, Sattelle DB. Novel animal-health drug targets from ligand-gated chloride channels. *Nat. Rev. Drug Discov.* 1(6), 427–436 (2002).
- 124 Stehr CM, Linbo TL, Incardona JP, Scholz NL. The developmental neurotoxicity of fipronil: notochord degeneration and locomotor defects in zebrafish embryos and larvae. *Toxicol. Sci.* 92(1), 270–278 (2006).
- 125 Cleland TA. Inhibitory glutamate receptor channels. *Mol. Neurobiol.* 13(2), 97–136 (1996).
- 126 Ihara M, Ishida C, Okuda H, Ozoe Y, Matsuda K. Differential blocking actions of 4'-ethynyl-4-n-propylbicycloorthobenzoate (EBOB) and gamma-hexachlorocyclohexane (gamma-HCH) on gamma-aminobutyric acid- and glutamate-induced responses of American cockroach neurons. *Invert. Neurosci.* 5(3–4), 157–164 (2005).
- 127 Silva MR, Oliveira CA, Felicio LF, Nasello AG, Bernardi MM. Perinatal treatment with picrotoxin induces sexual, behavioral, and neuroendocrine changes in male rats. *Pharmacol. Biochem. Behav.* 60(1), 203–208 (1998).
- 128 Silva MH, Gammon D. An assessment of the developmental, reproductive, and neurotoxicity of endosulfan. *Birth Defects Res. B Dev. Reprod. Toxicol.* 86(1), 1–28 (2009).
- 129 Kuperman RG, Checkai RT, Simini M, Phillips CT, Kolakowski JE, Lanno R. Soil properties affect the toxicities of 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) to the enchytraeid worm *Enchytraeus crypticus*. *Environ. Toxicol. Chem.* 32(11), 2648–2659 (2013).
- 130 Robidoux PY, Hawari J, Bardai G *et al.* TNT, RDX, and HMX decrease earthworm (*Eisenia andrei*) life-cycle responses in a spiked natural forest soil. *Arch. Environ. Contam. Toxicol.* 43(4), 379–388 (2002).
- 131 Cote I, Anastas PT, Birnbaum LS *et al.* Advancing the next generation of health risk assessment. *Environ. Health Perspect.* 120(11), 1499–1502 (2012).
- 132 Ellis P, Fowler P, Booth E *et al.* Where will genetic toxicology testing be in 30 years' time? Summary report of the 25th Industrial Genotoxicity Group Meeting, Royal Society of Medicine, London, November 9, 2011. *Mutagenesis* 29(1), 73–77 (2014).
- 133 van Zelm R, Huijbregts MA, Harbers JV *et al.* Uncertainty in msPAF-based ecotoxicological effect factors for freshwater ecosystems in life cycle impact assessment. *Integr. Environ. Assess. Manag.* 3(2), 203–210 (2007).
- 134 National Research Council (US) Committee on Applications of Toxicogenomics to Cross-Species Extrapolation. Application of toxicogenomics to cross-species extrapolation: a report of a workshop (2005).