How Theories Evolved Concerning the Mechanism of Action of Barbiturates

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Summary

The barbiturate phenobarbital has been in use in the treatment of epilepsy for 100 years. It has long been recognized that barbiturates act by prolonging and potentiating the action of γ-aminobutyric acid (GABA) on GABA_A receptors and at higher concentrations directly activating the receptors. A large body of data supports the concept that GABA_A receptors are the primary central nervous system target for barbiturates, including the finding that transgenic mice with a point mutation in the β3 GABA_A-receptor subunit exhibit diminished sensitivity to the sedative and immobilizing actions of the anesthetic barbiturate pentobarbital. Although phenobarbital is only modestly less potent as a GABA_A-receptor modulator than pentobarbital, phenobarbital is minimally sedating at effective anticonvulsant doses. Possible explanations for the reduced sedative effect of phenobarbital include more regionally restricted action; partial agonist activity; reduced propensity to directly activate GABA_A receptors (possibly including extrasynaptic receptors containing δ subunits); and reduced activity at other ion channel targets, including voltage-gated calcium channels. In recent years, substantial progress has been made in defining the structural features of GABA_A receptors responsible for gating and allosteric modulation by drugs. Although the precise sites of action of barbiturates have not yet been defined, the second and third transmembrane domains of the β subunit appear to be critical; binding may involve a pocket formed by β-subunit methionine 286 as well as α-subunit methionine 236. In addition to effects on GABA_A receptors, barbiturates block AMPA/kainate receptors, and they inhibit glutamate release through an effect on P/Q-type high-voltage activated calcium channels. The combination of these various actions likely accounts for their diverse clinical activities. Despite the remarkable progress of the last century, there is still much to learn about the actions of barbiturates that can be applied to the discovery of new, more therapeutically useful agents.

Key Words: Phenobarbital, Pentobarbital, Epilepsy, Seizures, GABA_A receptors.

The history of the barbiturates started with the synthesis of barbituric acid (malonylurea) by the German chemist and Nobel Prize winner Adolf von Baeyer (Fig. 1A) in 1864. Although barbituric acid itself does not exert any effects on the central nervous system (CNS), probably because it is not sufficiently lipophilic to penetrate the blood–brain barrier (Fig. 1A), it formed the backbone for all subsequently developed CNS-active barbiturates. In 1903, two German chemists working at the Bayer company, Emil Fischer (Fig. 1B) and Joseph von Mering, discovered that the more lipophilic barbituric acid derivative barbital (5,5-diethylbarbituric acid) was very effective in inducing sleep in dogs (Cozanitis, 2004). The same group synthesized phenobarbital (5-ethyl-5-phenylbarbituric acid; Fig. 1B) in 1911, and both barbital (Veronal) and phenobarbital (Luminal) were soon marketed by Bayer as hypnotic drugs for patients with insomnia. A shorter-acting hypnotic, pentobarbital (5-ethyl-5-(1-methylbutyl)barbituric acid; Neodorm; Fig. 1D), was also developed at Bayer and patented in 1916.
In 1912, the German psychiatrist Alfred Hauptmann (Fig. 1C) discovered the anticonvulsant activity of phenobarbital, which rapidly supplanted bromides as an effective and safe treatment for epilepsy. Based on the large medical success of these first barbiturates, >2,500 barbiturates were synthesized in following decades, some 50 of which were eventually employed clinically. In addition to their use as orally active sedative/hypnotic and antiepileptic drugs, parenteral administration of water soluble sodium salts of more lipophilic barbiturates was developed for intravenous anesthesia. The first barbiturate to be used systematically in anesthesia was sodium sec-butyl-(2-bromo-allyl)-barbiturate (Pernocton), which was introduced into the field by the German obstetrician Bumm in 1927. Notable among the pioneers in this field is John S. Lundy of the Mayo Clinic (Rochester, MN, U.S.A.), who introduced sodium amobarbital (1929) and sodium pentobarbital (1930; Nembutal) in anesthesia (Lopez-Munoz et al., 2005). By addition of a sulfur group to pentobarbital, lipophilicity was markedly enhanced, leading to the development of short-acting thiobarbiturates for anesthesia in 1935. The differential pharmacokinetic properties of these agents made it possible to draw up a practical clinical classification, based on the duration of their pharmacologic action (Lopez-Munoz et al., 2005). Therefore, the barbiturates in the category of short or intermediate action (secobarbital, amobarbital, pentobarbital) were employed initially as hypnotics, whereas those of prolonged action (phenobarbital) were widely used as anxiolytics and anticonvulsants; ultrashort-acting agents, notably sodium thiopental, were especially useful as anesthetics for minor operations. Despite their widespread use during the first half of the 20th century, no barbiturate succeeded in eliminating the main drawbacks of these drugs, which were sedative side effects, physical dependence during prolonged treatment (which can lead to addiction), exacerbation of seizures upon withdrawal, and potentially lethal effects of overdose. This led to a marked decline of barbiturate therapy in recent decades, particularly after the clinical introduction of the much safer benzodiazepines at the end of the 1950s. However, although only few barbiturates remained on the market, phenobarbital is still widely used as an anticonvulsant and sedative/hypnotic drug, particularly in developing countries (Kwan & Brodie, 2004).

The different pharmacologic effects of barbiturates depend on the administered dose and the resultant CNS depression with increasing brain concentrations. Therefore, with increasing doses all barbiturates induce anticonvulsant and anxiolytic activity, sedation, hypnosis, general anesthesia, and, at overdosage, death by respiratory depression.

Figure 1.
(A) The German chemist and Nobel laureate Adolf von Baeyer, who synthesized barbituric acid in 1864. (B) The German chemist and Nobel Laureate Emil Fischer, who synthesized phenobarbital (5-ethyl-5-phenylbarbituric acid) as a hypnotic drug at Bayer in 1911. (C) The German psychiatrist Alfred Hauptmann, who discovered the anticonvulsant activity of phenobarbital in 1912. (D) Pentobarbital [5-ethyl-5-(1-methylbutyl)-barbituric acid], which was developed as a hypnotic drug at Bayer and, since 1930, was also used in the form of its water-soluble sodium salt for intravenous anesthesia. Note the much higher lipophilicity (logP) of phenobarbital and pentobarbital versus barbituric acid.

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which is the basis for the use of barbiturates in veterinary and human euthanasia and capital punishment. These diverse pharmacologic effects of barbiturates represent a hurdle when studying mechanisms of action of a specific pharmacologic effect, such as seizure protection or anesthesia. One strategy that has been used to differentiate between anticonvulsant and anesthetic mechanisms is to study phenobarbital and pentobarbital in vitro systems at concentrations relevant to the specific behavioral action of interest. Although both barbiturates exert qualitatively the same pharmacologic effects at increasing concentrations, pentobarbital is about 13 times more lipophilic than phenobarbital, so that the anesthetic effect (with loss of righting reflexes in rodents) is achieved >10 times more rapidly after intravenous injection compared to phenobarbital. At anticonvulsant doses, phenobarbital is less sedative and has a higher therapeutic index than pentobarbital, which may be explained by its lower capacity to produce profound depression of neuronal function as compared with the anesthetic barbiturates. These differences cannot be explained by differences in lipid solubility alone.

**How Theories Evolved Concerning the Mechanism of Action of Barbiturates**

Numerous extensive reviews on mechanisms of action of barbiturates have been published (Nicoll, 1978; MacDonald & Barker, 1979; Prichard, 1980; Ho & Harris, 1981; Jurna, 1985; Rogawski & Porter, 1990; Prichard & Ransom, 1995; Harrison et al., 2000; Olsen, 2002; Rogawski & Lösch, 2004; Porter et al., 2012). Herein we briefly summarize the main findings with emphasis on phenobarbital and pentobarbital. It should be noted that most of the early studies on synaptic transmission (and membrane excitability) were performed at high, often therapeutically irrelevant, concentrations of barbiturates. To our knowledge, the concept of studying mechanisms of barbiturates in in vitro preparations at concentrations considered to be therapeutically relevant in humans was first used in the 1970s and 1980s (Nicoll, 1978; MacDonald et al., 1985).

**Early Studies**

Not many studies on mechanisms of action of barbiturates were performed before the 1940s (cf., Lundy & Osterberg, 1929; Tatum, 1939, 1940). It was soon recognized that the brain is the chief site of action of barbiturates. Based on early studies indicating a specific distribution of barbiturates in the brain, it was proposed that the anesthetic effect is mediated via the brain stem, whereas the sedative/hypnotic effect is mediated via a “sleep center” in the thalamus (Keeser & Keeser, 1927). However, subsequent studies showed ubiquitous distribution of barbiturates in the brain (Koppanyi & Dille, 1935).

A number of groups reported that the depressive effects of barbiturates on the CNS can be antagonized by the central stimulants picrotoxin and pentylentetrazole (PTZ; metrazol; Cardiazol; Maloney et al., 1931; Zipf et al., 1937; Hjort et al., 1938). This soon became a clinical treatment for barbiturate poisoning and was used to terminate barbiturate-induced general anesthesia (“awakening effect”) (Jones et al., 1950). At the time of these findings, it was not known that PTZ and picrotoxin act as antagonists of the neurotransmitter γ-aminobutyric acid (GABA).

In the 1940s and 1950s, it was increasingly recognized that barbiturates act by affecting membrane excitability and synaptic transmission (cf., Jurna, 1985). Following the discovery of GABA in the brain in 1950 and its establishment as a major inhibitory neurotransmitter in the subsequent two decades, the potential role of GABA in the mechanism of action of barbiturates was soon investigated. However, although effects on GABA-mediated inhibitory synaptic transmission appear to account, at least in part, for the sedative, hypnotic, anticonvulsant, and anesthetic actions of clinically used barbiturates, which will be discussed in more detail below, certain barbiturates have divergent actions that are not fully understood.

**Divergent Actions of Some Barbiturates**

A principal divergence relates to the unique ability of phenobarbital to protect against seizures at minimally sedating doses. The anesthetic barbiturate pentobarbital is highly effective as an anticonvulsant (ED\(_{50}\) in mouse maximal electroshock seizure test, 19.3 mg/kg) but it causes motor impairment at anticonvulsant doses (TD\(_{50}\), 18.9 mg/kg) (Raines et al., 1979). In contrast, phenobarbital has similar potency to pentobarbital in the seizure test (ED\(_{50}\), 22.5 mg/kg), but it is less potent in causing sedation (TD\(_{50}\), 61 mg/kg). These animal studies correspond with what is known from clinical experience. The range of free pentobarbital concentrations that produce anticonvulsant activity in man overlaps that causing anesthesia (Schober et al., 2010). In contrast, phenobarbital is an effective anticonvulsant drug that often produces only minimal sedation at therapeutic doses (Kwan & Brodie, 2004).

Another striking difference among barbiturates is the convulsant effects found in certain derivatives with bulky alkyl groups (Ho & Harris, 1981). For two of these compounds, N-methyl-5-propyl-5-phenylbarbiturate and 5-(1,3-dimethylbutyl)-5-ethyl barbituric acid (DMBB), the (+) isomers are convulsants, whereas the (−) isomers are anesthetics (Downes et al., 1970; Büch et al., 1973). It
has been suggested that the stereoselective effects of DMBB are due to differences in the formation of hydrogen bonds at receptor sites (Ho & Harris, 1981). The stereoisomers of a number of other barbiturates also have been shown to differ in their effects, with the S(−) isomer generally being more potent than the R(+) isomer in producing anesthesia or death. Of interest, this stereospecificity applies to both the actions on GABA responses and calcium currents so that the stereospecificity cannot be used to determine which target is more relevant to pharmacologic activity (Akaike et al., 1985; ffrench-Mullen et al., 1993).

Unfortunately, most comparisons of barbiturate stereoisomers or enantiomers have been restricted to anesthetic and lethal effects. One exception is the observation that the two enantiomers of secobarbital are equipotent as anesthetic and is more toxic than the R(+) isomer (Haley & Gidley, 1970). It would be interesting to compare the behavioral and physiologic actions of other barbiturate stereoisomer pairs.

**Effects of Barbiturates on Neuronal Membrane Excitability**

As could be expected from their CNS-depressing activity, barbiturates reduce neuronal membrane excitability in various invertebrate and vertebrate preparations (cf., Jurna, 1985). For instance, at high concentrations, pentobarbital and phenobarbital were shown to block the generation of action potentials in nerve fibers (Heinbecker & Bartley, 1940; Schoepfle, 1957; Vazquez et al., 1975; Sabelli et al., 1977). Phenobarbital was demonstrated to reduce repetitive spike discharges in frog nerve fibers (Toman, 1949, 1952) and to hyperpolarize neuronal membranes (Takeuchi, 1968). Furthermore, phenobarbital enhances spike frequency adaptation by activating a slow outward current in neurons of the sea slug *Aplysia* (Cote et al., 1978; Huguenard & Wilson, 1985). However, in mouse neurons, phenobarbital was shown to limit sustained repetitive firing only in concentrations above the therapeutic range (Macdonald et al., 1985). In lobster axons, Blaustein (1968) observed that high concentrations of the barbiturates pentobarbital (6 mM) and thiopental (approximately 2 mM) reduce voltage-dependent sodium and potassium conductance. Barbiturates including pentobarbital and phenobarbital are weak acids that can lose a proton to form a resonance-stabilized anion in which the negative charge is delocalized around the molecule. Blaustein found that the blocking activity of pentobarbital occurred at pH 8.5 but not at pH 6.7. Because the pKa of pentobarbital is 8.0, the results indicated that the anion form is the active species. A similar result was obtained for block of voltage-dependent calcium channels (ffrench-Mullen et al., 1993). However, Narahashi et al. (1971) concluded that the unionized species is responsible for block of excitability in the squid giant axon.

It has been proposed that barbiturates, as was believed to be the case for other anesthetic agents, act by dissolving in the lipid bilayer of the neuronal membrane perturbing the function of membrane ion channels (cf., Ho & Harris, 1981). This theory is now outdated and it is believed that barbiturates as well as inhalation anesthetic agents interact directly and in a specific fashion with membrane proteins, most importantly Cys-loop receptors such as GABA_A receptors.

**Effects of Barbiturates on Synaptic Transmission**

Various early studies demonstrated that barbiturates depress physiologic excitations and enhance inhibitions, but these observations did not indicate whether barbiturates were acting presynaptically, to augment or depress transmitter release, or postsynaptically, to increase or decrease transmitter efficiency (Prichard & Ransom, 1995). Of interest, some early studies found differences between phenobarbital and pentobarbital. Thus, it was reported that pentobarbital inhibits synaptic transmission (Heinbecker & Bartley, 1940; Larrabee & Posternak, 1952) and blocks calcium uptake, whereas phenobarbital had little effect (Blaustein & Ector, 1975; Blaustein, 1976). However, both pentobarbital and phenobarbital blocked excitatory synaptic transmission at central synapses (Ransom & Barker, 1975; Macdonald & Barker, 1978). Furthermore, both drugs facilitated synaptic inhibition by GABA, which was blocked by picrotoxin (Eccles et al., 1963; Nicoll, 1975a, b; Nicoll et al., 1975). Nicoll, on the basis of extracellular field recordings in the rabbit olfactory bulb, proposed in 1972 that various anesthetics, including pentobarbital, act by prolonging postsynaptic inhibition at inhibitory synapses (see also Schofield, 1978). He concluded that prolongation of GABAergic inhibition in the CNS “might contribute to an agent’s ability to produce general anaesthesia.” Soon after these pioneering observations, it became clear that GABA receptors are a key target mediating the different pharmacologic and clinical effects of barbiturates.

**Effects of Barbiturates on GABA_A Receptors**

Our current understanding of how barbiturates interact with GABA receptors is largely based on the landmark studies of Bob Macdonald and Richard Olsen in the late 1970s and early 1980s (Fig. 2). Macdonald and Barker (1977, 1978) showed that pentobarbital and, less potently, phenobarbital enhance postsynaptic GABA responses. Single channel recordings of barbiturate-enhanced single
GABA_A receptor currents have demonstrated that phenobarbital and pentobarbital increase the mean channel open duration of GABA_A receptor currents without altering channel conductance or opening frequency (Macdonald & Twyman, 1991; Fig. 2B). Olsen’s group showed that barbiturates affect GABA responses by binding to an allosteric site on the GABA_A receptor and proposed that “this barbiturate binding site has a specificity that correlates well with the anesthetic and GABA-enhancing actions of these drugs and, thus, can be designated as a “barbiturate receptor” (Leeb-Lundberg et al., 1980; Fig. 2D). Through this site, barbiturates increase the open time of the chloride channel activated by GABA (Schulz & Macdonald, 1981; Dunwiddie et al., 1986). Furthermore, at high, anesthetic doses, barbiturates directly activate the chloride channel (Mathers & Barker, 1980; Yang & Olsen, 1987; Rho et al., 1996). This is an important difference from benzodiazepines, which act by way of a distinct binding site at the GABA_A receptor (Figs 2D and 3) to augment the frequency of chloride channel opening (Fig. 2B), but do not directly activate the channel in the absence of GABA. These differences are one factor that explains the much higher safety of benzodiazepines compared to barbiturates (Olsen, 2002). Another factor is that benzodiazepines produce a limited increase in chloride current, whereas barbiturates increase the current many fold (Kokate et al., 1994). Recently, it has been shown that a benzodiazepine and the barbiturate pentobarbital (at low modulatory concentrations) induce different molecular movements in the receptor, further supporting the concept that the mechanisms underlying positive allosteric modulation by barbiturates and benzodiazepines are different (Sancar & Czajkowski, 2011). Finally, benzodiazepines act exclusively on GABA_A receptors, whereas barbiturates have many additional targets in the brain and periphery (including the heart) (see below).
The crucial role of GABA<sub>Α</sub> receptors in mediating pharmacologic effects of barbiturates has been elegantly demonstrated through the use of mice with a mutation (N2650M) in the β3 subunit of the GABA<sub>Α</sub> receptor that abolishes the ability of pentobarbital to directly activate the receptor and reduces pentobarbital modulation of GABA responses (Pistis et al., 1999). The mutation diminishes the hypnotic and immobilizing action of pentobarbital but interestingly not the respiratory depression, suggesting that this latter action may not depend upon GABA<sub>Α</sub> receptors (Zeller et al., 2007).

There are interesting differences in the effects of anticonvulsant and anesthetic barbiturates on GABAergic transmission (for review see Olsen, 2002; and Porter et al., 2012). Phenobarbital is modestly less potent than pentobarbital in enhancing GABA responses (half maximal effective concentration [EC<sub>50</sub>] values to enhance GABA-activated chloride current in hippocampal neurons, 12 µM vs. 3.4 µM, respectively; ffrench-Mullen et al., 1993; see also, Mathers et al., 2007). However, as noted previously, at anticonvulsant doses phenobarbital is less sedative than pentobarbital, which is difficult to explain by assuming that both drugs act as full agonists at the same type of GABA<sub>Α</sub> receptors. One explanation for this important difference may be that the two structurally distinct barbiturates influence different GABA<sub>Α</sub>-receptor subtypes expressed in different brain regions with different potencies. Thus, it has been found in some experiments that pentobarbital enhances GABA binding in all brain regions, whereas phenobarbital enhances GABA binding only in some regions (Olsen, 2002). Indeed, a recent study found that although pentobarbital affected GABA<sub>Α</sub> receptor responses in both neocortex and thalamus, phenobarbital was selective for neocortex and had effects on thalamic neurons only at very high, potentially toxic doses (Mathers et al., 2007). Nonselective depression of neocortex, thalamus (and other areas) by pentobarbital, and more selective depression of the cortex by phenobarbital could potentially be a factor to explain the relative lack of CNS depression produced by phenobarbital. As of yet, a coherent explanation of these differential actions in terms of the differing sensitivity of regionally expressed GABA<sub>Α</sub> receptors isoforms is not available. An alternative explanation is that the maximal enhancement of GABA<sub>Α</sub> receptor chloride current produced by phenobarbital is less than that of pentobarbital, so that phenobarbital can be described as a "partial agonist" compared to the full agonist pentobarbital (ffrench-Mullen et al., 1993; Rho et al., 1996). More generally, as discussed below, barbiturates have at least three actions on GABA<sub>Α</sub> receptors that are likely mediated by binding to distinct sites on the receptor complex. There may be differences in the relative activity of phenobarbital and pentobarbital at these sites that account for their pharmacologic differences. In addition to the possibility that phenobarbital is a partial agonist, the drug may also cause channel blocking effects that limit the degree to which it activates GABA<sub>Α</sub>-receptor chloride currents (see below). However, phenobarbital only causes this effect at millimolar concentrations that are even greater than those required for pentobarbital block.

GABA<sub>Α</sub> receptors are chloride channels that are members of the Cys-loop family of ligand-gated ion channels. They are heteropentamers composed of various combinations of five homologous subunits that are drawn from several subunit classes (e.g., α, β, and γ) (Miller & Smart, 2010; Sieghart et al., 2012). The existence of 19 different subunits gives rise to a multiplicity of GABA<sub>Α</sub>-receptor subtypes with distinct regional, cellular, and subcellular distributions, and diverse pharmacologic properties (Sieghart et al., 2012). Most GABA<sub>Α</sub> receptors in the CNS are composed of two α, two β, and one γ2 subunits arranged in a barrel-like manner (β-α-β-α-γ counterclockwise sequence) around a central channel axis that coincides with the ion permeation pathway (Fig. 3). The transmembrane ion permeation pathway is lined by the five α-helical M2 segments of each subunit. This inner ring, forming the wall of the channel pore, is surrounded by an outer ring of alternating M1 and M3 segments. The M4 segments are also a component of the outer ring. Whereas GABA acts at the two extracellular β-α interfaces, the allosteric modulatory benzodiazepines interact with the extracellular x-γ2 interface (Fig. 3).

Barbiturates, including pentobarbital and phenobarbital, have three distinct actions on GABA<sub>Α</sub> receptors. At low micromolar concentrations, phenobarbital and pentobarbital have little or no effect on their own but they enhance the activity of submaximal concentrations of GABA (ffrench-Mullen et al., 1993). At higher concentrations, pentobarbital and phenobarbital can activate GABA<sub>Α</sub> receptors in the absence of GABA (EC<sub>50</sub> values, 0.33 and 3 mM, respectively). At the highest concentrations (>1 mM for pentobarbital and >3 mM for phenobarbital), current flow through the channel is inhibited (Rho et al., 1996; Akk & Steinbach, 2000). Although the specific sites on the receptor complex subserving these various actions have not been identified, in general terms the effects of barbiturates depends in a major way on the β subunit, although agonist activity is influenced by the α subunit (Bureau & Olsen, 1993; Mathews et al., 1996; Thompson et al., 1996; Serafini et al., 2000; Drafts & Fisher, 2006; Zeller et al., 2007). However, certain mutations in β subunits that eliminate GABA gating of the receptor do not affect pentobarbital responses (Amin & Weiss, 1993). Similarly, photochemical cleavage of a specific region of the α-subunit (loop A-E linker) eliminates GABA activation of α1β2 GABA<sub>Α</sub> receptors, but does not affect activation by pentobarbital (Hanek et al., 2010). Moreover, functional GABA<sub>Α</sub> receptors can be formed entirely of β subunits, which are unresponsive to GABA but can be activated by pentobarbital (Cestari et al., 1996).
Figure 3.
The inhibitory synapse and the structure of the GABA<sub>A</sub> receptor illustrating some presumed sites of barbiturate action. (A1) Schematic illustration of a GABAergic synapse showing synthesis, vesicular packaging, release, uptake, and degradation of GABA in GABAergic nerve terminals; uptake into astrocytes; and the pentameric subunit structure of a typical GABA<sub>A</sub> receptor complex in the postsynaptic membrane. Components of the GABAergic synapse shown including glutamic acid decarboxylase (GAD), the enzyme that catalyzes the decarboxylation of glutamate to GABA; GABA-containing synaptic vesicles (circles containing GABA molecules); the GAT-1 GABA transporter (cylinders); and conversion of GABA to succinic semialdehyde (SSA) by GABA transaminase (GABA-T). (A2) Schematic of the pentameric structure within the plane of the membrane showing the relative positions of the transmembrane domains. Subunit interfaces are formed by M3 and M1. The interfacial locations of the two GABA and one benzodiazepine (BZ) recognitions sites are shown. Each protein subunit bas the topology represented in the schematic shown in A3 with a large extracellular N-terminal domain, four transmembrane domains (M1–M4), and a large cytoplasmic loop between M3 and M4. M2 forms the wall of the channel pore. Barbiturates are believed to interact with the M2 and M3 β-subunit domains. (B1) Ribbon diagram of the α and β subunits, with a detailed view of the subunit interface in B2. The extracellular binding domain is colored white. Domains believed to contribute to the GABA transduction mechanism (loop 2, loop 7, M2–M3 linker, and pre-M1) are highlighted in yellow. The loop C region of the GABA binding site is highlighted in green. The transmembrane domains are colored in red; M4 domains are omitted for clarity. Residues in the pre-M1 region (R216) as well as residues forming the potential barbiturate binding site (N265 and M286) in the β subunit and M236 in the α subunit are shown in a space-filled format. Adapted from Mercado and Czajkowski (2008). In the model of Olsen and Li (2011) based on photoaffinity labeling with etomidate, αM1-M236 is considered close to βM3-M286 and forms the general anesthetic binding site.
Therefore, \(\beta\) subunits contain a recognition site sufficient for barbiturate binding, but GABA binding requires the engagement of \(\alpha\) subunits. Moreover, it is apparent that the agonist site at which barbiturates interact with \(\text{GABA}_A\) receptors to induce a chloride current response is distinct from the site at which GABA binds to open the channel.

Of interest, the unitary single channel conductance activated by GABA and barbiturates is similar, suggesting that the structure of the open channel activated by the two agonists is similar (Rho et al., 1996). However, recent studies indicate that the structural movements in the channel protein induced by GABA and pentobarbital that are associated with channel gating are different, indicating that they activate the \(\text{GABA}_A\) receptor by different mechanisms (Mercado & Czajkowski, 2008; Muroi et al., 2009; Eaton et al., 2012). A stretch of amino acids in the extracellular domain immediately before the M1 transmembrane domain (pre-M1) may act as a “central hub” coupling GABA-induced motions in the GABA binding site to a sequence of movements in extracellular domain that trigger movements in the M2 channel region that opens the channel. Mutation in the pre-M1 domain of either the \(\alpha\) or \(\beta\) subunit was found to abolish gating by both GABA and pentobarbital, suggesting that the pre-M1 region plays a role in activation of the channel by both ligands (Mercado & Czajkowski, 2006). However, a recent study indicates that pentobarbital triggers different movements than GABA in the \(\alpha\) and \(\beta\) pre-M1 regions (Mercado & Czajkowski, 2008). Mutations in M2 of the \(\beta\) subunit can also eliminate pentobarbital activation of the receptor, although modulation is preserved (Pistis et al., 1999), whereas mutations in M3 also influence pentobarbital modulation of the receptor (Amin, 1999). Similarly, a mutation in the extracellular domain of the \(\gamma2\) subunit (S195C) influences gating by GABA or the agonist muscimol but not pentobarbital and etomidate (Eaton et al., 2012).

A series of studies with a photo-incorporable analog of the general anesthetic etomidate has led to the identification of residues in the M1 domain of the \(\alpha\) subunit (M236) and M3 domain of the \(\beta\) subunit (M286) that were considered adjacent and components of a binding pocket for etomidate and possibly also barbiturates (Olsen & Li, 2011). Recently, V290 in the \(\beta\)-subunit has also been identified as a residue critical to etomidate binding (Chiara et al., 2012). It is noteworthy that the interface between the \(\alpha\) and \(\beta\) subunits also contains the GABA binding site in the extracellular domain. It has been argued that this binding site is relevant to both the GABA potentiating and direct gating of the \(\text{GABA}_A\) receptor (Li et al., 2006). Indeed, there is evidence that, at least for etomidate, low occupancy of two equivalent binding sites is responsible for the enhancing action, whereas high occupancy directly gates the channel (Rüsch et al., 2004). Although etomidate and barbiturates both enhance GABA responses and directly activate \(\text{GABA}_A\) receptors, the binding sites may not be identical as potentiation of GABA responses by the two modulators exhibit pharmacologic differences (Akk et al., 2011).

To define the precise location for binding of barbiturates, x-ray crystal structures of the \(\text{GABA}_A\)-receptor channels may be required. To date, the crystal structure of the mammalian \(\text{GABA}_A\) receptors has not been solved. However, high-resolution crystal structures of several homologous channels are available. One of these is the bacterial \textit{Gloeobacter violaceus} pentameric ligand-gated ion channel (GLIC), which is a proton-activated channel that is a structural homolog of Cys-loop receptors (Bocquet et al., 2009). The barbiturate thiopental was found to bind to an \textit{intersubunit} pocket, as was the case for mammalian \(\text{GABA}_A\) receptors (Chen et al., 2010).

In addition to \(\alpha\beta\gamma\delta\) \(\text{GABA}_A\) receptors that are localized to synapses, a second major class of \(\text{GABA}_A\) receptors in the brain are composed of \(\alpha\beta\delta\) subunits; these are believed to be preferentially located at extrasynaptic or perisynaptic sites, where they mediate tonic inhibition. Even high concentrations of GABA induce only small currents in \(\text{GABA}_A\) receptors that contain \(\delta\) subunits, which is interpreted as indicating that GABA is a “partial agonist” at this \(\text{GABA}_A\)-receptor subtype. However, such receptors are highly susceptible to positive modulation by allosteric potentiators such as barbiturates (Adkins et al., 2001; Feng et al., 2004). In addition, they seem highly sensitive to direct gating by pentobarbital (Feng et al., 2004). Moreover, in experiments with either native \(\delta\) subunits or \(\delta\) subunit containing concatemeric receptors engineered to provide good expression, pentobarbital caused a markedly greater enhancement of the maximum current generated by saturating GABA than was the case in \(\gamma2\) \(\text{GABA}_A\) receptors (Feng et al., 2004; Shu et al., 2012). It seems likely that effects on extrasynaptic \(\delta\)-subunit–containing \(\text{GABA}_A\) receptors are important for the pharmacologic actions of anesthetic barbiturates such as pentobarbital. As of yet, the precise way in which barbiturates modulate \(\delta\) \(\text{GABA}_A\) receptors is not known. However, the N-terminal extracellular domain and the N-terminal portion of the M1 domain of the \(\delta\)-subunit are required (Feng & Macdonald, 2010).

Of interest, chronic treatment with barbiturates causes an increase in \(\text{GABA}_A\)-receptor \(\delta\) subunit mRNA; withdrawal results in down-regulation (Lin & Wang, 1996). Therefore, the relative activity of barbiturates on synaptic and extrasynaptic \(\text{GABA}_A\) receptors may vary with drug exposure.

We recently used a new strategy to determine which \(\text{GABA}_A\) receptor subunits are important for the anticonvulsant effect of phenobarbital by comparing \(\text{GABA}_A\) receptor binding and subunit expression in phenobarbital-resistant and phenobarbital-responsive epileptic rats (Volk et al., 2006; Bethmann et al., 2008). In this model of temporal lobe epilepsy (TLE), spontaneous recurrent
seizures develop several weeks after status epilepticus that is induced by sustained electrical stimulation of the basolateral amygdala (Brandt et al., 2004). When such epileptic rats are treated with maximum tolerated doses of phenobarbital over 2 weeks, a subset of the animals ("responders") show almost complete suppression of seizures, whereas about 30–40% of the rats do not exhibit any significant response to treatment ("nonresponders"; Fig. 4A). The adverse effects (sedation, ataxia) of phenobarbital are the same in both subgroups; only the anticonvulsant effect is lost in the nonresponders. In a first study on GABA A receptors in this model, we found striking differences in GABA A-receptor sensitivity between phenobarbital responders and nonresponders, as demonstrated by a shift to diazepam-insensitive GABA A receptors in the dentate gyrus of nonresponders (Volk et al., 2006; Fig. 4B). A similar change to diazepam-insensitive GABA A receptors has previously been reported in the pilocarpine model of TLE, due to a shift in GABA A receptor subunits from α1 + β1 to α4 + δ (Brooks-Kayal et al., 1998), raising the possibility that phenobarbital nonresponders differ from responders in the subunit
expression of GABA<sub>A</sub> receptors. In fact, a subsequent study showed significant differences in GABA<sub>A</sub>-receptor subunit expression between phenobarbital responders and nonresponders; particularly widespread reduction in β1/2 and several α-subunits were found in nonresponders versus responders but there was up-regulation in the α4-subunit (Bethmann et al., 2008; Fig. 4C). These findings are likely involved in the resistance to phenobarbital in our epilepsy model.

It is important to note that the effects of phenobarbital on GABA<sub>A</sub> receptors are age dependent. In newborns, GABA depolarizes the neuron and may exert excitatory effects, instead of inhibitory effects as in adults (Holmes et al., 2002). This is a result of the high intraneuronal chloride concentrations early after birth. Therefore, GABA potentiators such as phenobarbital or diazepam may exacerbate seizures in neonates (Kahle & Staley, 2008). Depolarizing GABA effects have also been described in the hippocampus of adult patients with pharmacoresistant epilepsy and are thought to result from alterations in the cation-chloride cotransporters NKCC1 (a Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup> cotransporter) and KCC2 (a K<sup>+</sup>-Cl<sup>−</sup> cotransporter) (cf., Löschler et al., 2012). By reducing intracellular chloride, NKCC1 inhibitors such as bumetanide may enhance the action of phenobarbital. The drug combination may provide a new therapeutic approach, both for neonatal seizures and pharmacoresistant seizures in adult patients with epilepsy.

Furthermore, it is noteworthy that the many similarities in the pharmacology of barbiturates and ethanol are likely to be a consequence of their common interaction with GABA<sub>A</sub> receptors (Chen & Olsen, 2007). This also explains the cross-tolerance between ethanol and barbiturates that develops during chronic treatment (Kumar et al., 2004) and the historical utility of barbiturates in the treatment of alcohol withdrawal symptoms (Erstad & Cotugno, 1995).

**Other Potentially Relevant Mechanisms of Barbiturates**

In contrast to benzodiazepines, which are highly specific in their action, barbiturates do not only enhance GABA responses via an interaction with the GABA<sub>A</sub>...
receptor, but exert several other effects on synaptic function and intrinsic neuronal excitability mechanisms that may be involved in their pharmacologic actions. Therefore, barbiturates diminish glutamate responses (Macdonald & Barker, 1978) and phenobarbital has been shown to block the non-NMDA (AMPA/kainate) subtype of glutamate receptors at anticonvulsant doses (Frandsen et al., 1990; Ko et al., 1997; Nardou et al., 2011).

In addition to effects on ligand-gated channels, barbiturates have been shown to inhibit some voltage-activated ion channels, including voltage-activated potassium (Warthenberg et al., 2001; Lingamaneni & Hemmings, 2003) and calcium channels (Wertz & Macdonald, 1985; ffrench-Mullen et al., 1993; Schober et al., 2010). The effect on sodium channels is unlikely to be relevant to the anticonvulsant activity of phenobarbital (Lingamaneni & Hemmings, 2003) or the anesthetic activity of pentobarbital (Baudoux et al., 2003; Lingamaneni & Hemmings, 2003). However, blockade of calcium channels is likely to be a factor. Phenobarbital and pentobarbital inhibit high-voltage activated calcium currents (ffrench-Mullen et al., 1993) at concentrations within the relevant free therapeutic plasma concentrations ranges (clinical anticonvulsant plasma levels of phenobarbital, 43–194 μM total, 40–50% of this is free; Eadie & Kwan, 2008; anesthetic concentrations of pentobarbital, 50–80 μM). P/Q-type high-voltage activated calcium channels (CaV2.1) are the main calcium channel type responsible for the entry of calcium into presynaptic nerve terminals. Activation of these channels is required for release of neurotransmitters, including the main excitatory transmitter glutamate. A recent study found that pentobarbital causes slow, open channel block of recombinant human P/Q-type calcium channels (Schober et al., 2010). In line with this action, pentobarbital and also phenobarbital have been shown to inhibit glutamate release through effects on P/Q calcium channels (IC50 [half maximal inhibitory concentration] values, 22 and 112 μM, respectively; Kitayama et al., 2002). More directly, in hippocampal CA1 neurons, pentobarbital was shown to inhibit calcium transients in synaptic boutons and also the amplitude of excitatory synaptic potentials, the electrophysiologic consequences of glutamate release (Baudoux et al., 2003). Effects on calcium channels seem more likely to be of relevance to the anesthetic activity of pentobarbital than to the anticonvulsant activity of phenobarbital, which is a less-potent calcium channel blocker and less potent at inhibiting glutamate release (ffrench-Mullen et al., 1993; Kitayama et al., 2002). In addition, one study has observed that pentobarbital enhances inward rectifier potassium current in thalamic neurons, which could potentially contribute to its anesthetic action (Wan et al., 2003). It is apparent that the important divergence noted previously between phenobarbital and pentobarbital in terms of sedative activity at anticonvulsant doses is likely to be explained not only by the differences in the way the two drugs affect GABA A receptors but also due to differences in activity at other ion channel targets.

It is noteworthy that barbiturates have a negative ionotropic effect on the heart and can influence cardiac calcium (Gilat et al., 1987) and potassium channels (Bachmann et al., 2002) at concentrations that are near the anesthetic-free plasma concentrations, potentially producing adverse cardiac effects (Nattel et al., 1990). In addition, pentobarbital has been shown to inhibit neuronal nicotinic acetylcholine receptors, such as those in intracardiac ganglia neurons, so that effects on parasympathetic mechanisms could also perturb cardiac function (Tonner & Miller, 1995; Weber et al., 2005).

Conclusions

One hundred years after the discovery of the antiepileptic effect of phenobarbital by Hauptmann in 1912, the mechanism of its antiseizure action is still not completely understood. At least in part, this is due to the lack of specific barbiturate antagonists and the fact that barbiturates exert diverse effects on synaptic function apart from their potentiating action on GABAergic transmission. However, at clinically relevant concentrations, phenobarbital acts primarily as a GABA potentiator, although it is weaker than hypnotic/anesthetic barbiturates such as pentobarbital. Furthermore, hypnotic/anesthetic barbiturates such as pentobarbital act also on calcium channels and, at anesthetic levels, directly open GABA A receptor–associated chloride channels. In addition, effects on extrasynaptic GABA receptors may be involved in the sedative/hypnotic effects of barbiturates. Overall, potentiation of synaptic GABAergic transmission is the most likely mechanism of the anticonvulsant action of phenobarbital, but contributions of additional mechanisms such as blockade of non-NMDA (AMPA/kainate) receptors and voltage-activated calcium channels cannot be ruled out.

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Disclosure

None of the authors has any relevant conflicts of interest to disclose. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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