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REVIEW

Stress and $GABA_A$ receptors

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Abstract

$GABA_A$ receptors are sensitive to subtle changes in the environment in both early-life and adulthood. These neurochemical responses to stress in adulthood are sex-dependent. Acute stress induces rapid changes in $GABA_A$ receptors in experimental animals, with the direction of the changes varying according to the sex of the animals and the stress-paradigm studied. These rapid alterations are of particular interest as they provide an example of fast neurotransmitter system plasticity that may be mediated by stress-induced increases in neurosteroids, perhaps via effects on phosphorylation and/or receptor trafficking. Interestingly, some studies have also provided evidence for long-lasting changes in $GABA_A$ receptors as a result of exposure to stressors in early-life. The short- and long-term stress sensitivity of the GABAergic system implicates $GABA_A$ receptors in the non-genetic etiology of psychiatric illnesses such as depression and schizophrenia in which stress may be an important factor.

Keywords: depression, development, $GABA_A$ receptor, schizophrenia, sex differences, stress.

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Stress is defined as an integrated bodily response that is produced to deal with extraordinary circumstances. Both the short-term effects of stress on the adult nervous system and the long-lasting effects of stress on the developing nervous system are implicated in the onset of neuropsychiatric conditions such as anxiety disorders, schizophrenia, and depression. Given that stress has also been implicated in the pathology of such psychiatric disorders, the effects of stress on $GABA_A$ receptors may be relevant to our understanding of the molecular association between stress and psychiatric disorders. However, our understanding of the effects of acute stress on $GABA_A$ receptors is complicated by a number of conflicting findings in this area. Furthermore, while previous studies have indicated long-lasting effects of early-life stress on multiple neurotransmitter systems (Heim and Nemeroff 2001; Arborelius and Eklund 2007), the GABAergic system has largely been ignored, indicating a need for an improved understanding of the effects of early-life stress on adult $GABA_A$ receptors.

$GABA_A$ receptor composition

$GABA_A$ receptor ionophores are a complex receptor class. Combined affinity purification and cloning from cDNA libraries has identified 16 subunits from which $GABA_A$ receptor pentamers may be assembled. These subunits are encoded by separate genes and classified by sequence identity into seven subunit classes, including six α (α_1–α_6), four β (β_1–β_4), three γ (γ_1–γ_3, two splice variants; γ_{2short}, γ_{2long}), one δ, one ε, and one θ subunit (Whiting 2003). *In vitro* studies have shown that only certain subunit combinations may form functional receptors that reach the plasma membrane including αβ, αβγ/∂/ε/θ combinations (Pritchett *et al.* 1989; Connolly *et al.* 1996b; Gorrie *et al.* 1997; Kittler *et al.* 2000). Moreover, different subunits preferentially co-assemble. For example, δ subunits preferentially co-assemble with α_4 or α_6 subunits (Quirk *et al.* 1995; Jacob *et al.* 2008). Immunohistochemistry and *in situ* hybridization studies measuring subunit colocalization on membranes have supported these *in vitro* studies and shown that most $GABA_A$ receptor subtypes contain α, β, and γ subunits (Wisden *et al.* 1992; Fritschy and Mohler 1995; Sieghart *et al.*

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Abbreviations used: AFR, animal facility rearing; ED, embryonic day; EH, early handled; HPA, hypothalamic pituitary adrenal; MS, maternal separation; NH, non-handled; PKC, protein kinase C; PND, postnatal day; SS, somatosensory; TBPS, *t*-butylbicyclophosphorothionate; THDOC, allotetrahydrodeoxycorticosterone.

1999), particularly in the ratio 2 : 2 : 1, although stoichiometry may vary (i.e. 2 : 1 : 2, 3 : 2 : 0) (Whiting *et al.* 1995).

Different receptor subtypes vary in their pharmacological sensitivities, channel kinetics as well as their ontogenic, regional, and cellular distributions. Of recent interest is the different composition of $GABA_A$ receptors located at synaptic compared with extrasynaptic sites (Belelli *et al.* 2009). For example, receptors containing γ_2 subunits are predominantly clustered in synaptic locations, while receptors containing δ subunits are generally diffusely located at extrasynaptic sites (Essrich *et al.* 1998; Jacob *et al.* 2008). Receptors at extrasynaptic sites provide tonic inhibition as demonstrated by the slow decay kinetics and high affinity for GABA of δ-subunit containing $GABA_A$ receptors, allowing for sensitivity to GABA that spills over from the synapse (Saxena and Macdonald 1994; Banks *et al.* 2000). This tonic inhibition appears to serve an important role in brain function given that δ-subunit knockout mice display spontaneous seizures indicative of a drastic loss of inhibitory tone (Mihalek *et al.* 1999).

$GABA_A$ receptor pharmacology

The orthosteric site

The orthosteric site of $GABA_A$ receptors is the site where GABA binds to induce chloride channel opening and membrane currents. The orthosteric site is selectively blocked by the antagonist bicuculline but no selective $GABA_A$ receptor agonist exists that does not act on $GABA_B$ or $GABA_C$ receptors (Johnston 2005). For example, muscimol acts as a bicuculline-sensitive agonist at $GABA_A$ receptor orthosteric sites but also acts as a more potent bicuculline-insensitive agonist at $GABA_C$ receptors (Johnston 2005).

High- and low-affinity binding sites

The orthosteric binding site has been extensively studied using radiolabeled agonists such as [^{3}H]GABA and [^{3}H]muscimol and antagonists such as [^{3}H]bicuculline and [^{3}H]SR95531. Analysis of Scatchard's plots from such studies has lead to a general consensus that there exist both high-affinity (nM) and low-affinity (nM–μM) orthosteric binding sites. Whether these two different binding site populations represent different conformations of the same binding site, or distinct sites on the same or different macromolecular complexes is unknown (Harris and Allan 1985; Cash and Subbarao 1987; Edgar and Schwartz 1992; Smith and Olsen 1994; Maksay 1996; Baur and Sigel 2003; Yeung *et al.* 2003). However, electrophysiological studies on cerebellar neuronal patches (Maconochie *et al.* 1994) and recombinant receptors (Baur and Sigel 2003), as well as studies of chloride uptake into brain vesicle preparations (Harris and Allan 1985), all show that μM concentrations of GABA are required for channel opening, suggesting that the low-affinity GABA binding site represents the functional site.

Allosteric sites

$GABA_A$ receptors contain many allosteric modulatory sites that are presumably remote from the orthosteric site. When these sites are occupied, binding of GABA or its ability to open the ion channel changes. Agents that act to enhance the action of GABA on $GABA_A$ receptors are termed positive modulators and these compounds have widespread therapeutic uses as anxiolytics, sedative-hypnotics, anticonvulsants, and anesthetics (Johnston 2005). Separate positive modulatory sites exist for a variety of compounds including benzodiazepines, barbiturates, anesthetics, ethanol, cations (e.g. Zn^{2+}, Mg^{2+}, and Ca^{2+}), endogenous neurosteroids (e.g. allopregnanolone and allotetrahydrodeoxycorticosterone; THDOC), and certain dietary compounds (flavonoids, terpenes, and sage) (Johnston 2005). Conversely, agents that reduce the action of GABA on $GABA_A$ receptors are termed negative modulators or inverse agonists and these compounds have anxiogenic and convulsant effects and are thus of limited therapeutic use. Agents can also block the allosteric modulatory sites without exerting any effect on the chloride channel opening and these are termed neutralizing allosteric modulators of which flumazenil is an example at the benzodiazepine site (Johnston 1996). In addition, some compounds appear to bind directly within the ion channel to block $GABA_A$ receptor function such as picrotoxin, *t*-butylbicyclophosphorothionate (TBPS) and *t*-butylbicycloorthobenzoate (Squires *et al.* 1983).

$GABA_A$ receptor trafficking and phosphorylation

Altered expression of protein subunits on the plasma membrane may arise from quite rapid (3–10 min) trafficking processes (up-/down-regulation) (Wan *et al.* 1997; Washbourne *et al.* 2004; Thomas *et al.* 2005), or over longer periods (hours) may arise from alterations in protein synthesis (Connolly *et al.* 1999). The processes involved in the expression of a functional $GABA_A$ receptor on the plasma membrane and the factors that can affect this expression are becoming increasingly understood (reviewed in detail elsewhere e.g. Kittler and Moss 2001; Luscher and Keller 2004). Briefly, receptor subunit proteins are assembled into pentameric ion channels in the endoplasmic reticulum then transported via the Golgi apparatus to the plasma membrane (Connolly *et al.* 1996a; Kittler and Moss 2001). Receptors are removed from the membrane via endocytosis into clathrin-coated vesicles (Barnes 2000). Once in clathrin-coated vesicles receptors may be returned to the endosomal system where they are degraded or recycled to the plasma membrane (Kittler *et al.* 2000). Large pools of $GABA_A$ receptors appear to reside intracellularly in clathrin-coated

vesicles, from where they may be rapidly expressed on the membrane surface (Tehrani and Barnes 1997; Tehrani *et al.* 1997).

The surface expression of $GABA_A$ receptors appears to be affected by receptor phosphorylation (Kittler and Moss 2003). For example, manipulation of the function of protein kinase C (PKC) *in vitro* alters membrane expression of receptors, and unphosphorylated β and γ_2 subunits colocalize with the clathrin-adaptor protein 2 (AP2) protein found in clathrin-coated pits (Connolly *et al.* 1999; Filippova *et al.* 2000). Interestingly, neurosteroids can regulate the activity of PKC and thus the phosphorylation of $GABA_A$ receptors (Brussaard and Koksma 2003). This suggests that neurosteroid concentration could regulate the surface expression of $GABA_A$ receptor subunits (Brussaard *et al.* 2000; Fanscik *et al.* 2000; Hodge *et al.* 2002).

The influence of phosphorylation on $GABA_A$ receptor function is not limited to its effects on receptor trafficking, and may directly influence the function of $GABA_A$ receptors expressed on the surface. For example, kinase-induced alterations of the $GABA_A$ receptor phosphorylation state via the β or γ_2 subunits may affect channel opening as suggested by effects of protein kinase A, PKC, and tyrosine kinase on GABA-induced chloride currents (Moss *et al.* 1995; Moss and Smart 1996; McDonald *et al.* 1998; Brandon and Moss 2000; Brandon *et al.* 2000, 2002). Furthermore, PKC has been shown to potentiate benzodiazepine and TBPS binding but inhibit muscimol binding in a region specific manner in the brain (Oh *et al.* 1999). Constitutive PKC activity also appears necessary for the neurosteroid allopregnanolone to positively modulate $GABA_A$ receptors (Brandon *et al.* 2002), and allopregnanolone binding at $GABA_A$ receptors prevents PKC-induced inhibition of $GABA_A$ receptor currents (Brussaard *et al.* 2000). Thus, receptor phosphorylation appears to be a mechanism via which receptor function may be rapidly affected either directly, or via its effects on receptor trafficking.

Acute stress and $GABA_A$ receptors

Acute stress involves recruitment of the hypothalamic pituitary adrenal (HPA) axis and the sympathetic-adrenal medullary system and results in both rapid (minutes) and more delayed effects on target tissues. Not surprisingly then, studies of the effects of acute adulthood stress on $GABA_A$ receptors have focused on rapid changes in binding site expression, affinity and function rather than the delayed effects on altered protein and mRNA expression of subunits. Radioligand binding studies suggest rapid alterations in the GABAergic system occur in response to stress. These rapid alterations are of particular interest as they provide an example of fast neurotransmitter system plasticity in response to experience that may be mediated by either the release of endogenous GABAergic ligands (i.e. neuroactive steroids or neurosteroids) and/or rapid trafficking of $GABA_A$ receptors.

Effects of acute stress on $GABA_A$ receptor orthosteric binding sites

Studies measuring [^{3}H]GABA binding suggest that the availability of low-affinity binding sites (B_{max}) is rapidly affected following stress in a sex and paradigm specific manner (see Table 1), while the affinity (K_D) is not affected. Studies in males suggest that different stressors produce different effects, with acute swim stress producing no changes (Skerritt *et al.* 1981; Skilbeck *et al.* 2008), while foot shock stress (Biggio *et al.* 1981; Concas *et al.* 1985; Corda *et al.* 1985; Cuadra and Molina 1993) and stress from guillotine in handling-naïve rats (Biggio *et al.* 1981, 1984, 1987; Concas *et al.* 1985) reduced forebrain low-affinity [^{3}H]GABA binding. Apparent differences between different stressors may also arise from different laboratory stress protocols given that the presence of conspecifics during stress (Cuadra and Molina 1993; Skilbeck *et al.* 2008) and habituation of animals to experimenter handling (Biggio

Table 1 Stress-induced changes in $GABA_A$ receptor orthosteric site binding (B_{max})

Stress	Animal	Radioligand	Change	Reference
Swim (32°C, 3 min)	Male mice	[^{3}H]GABA, low affinity	No change	Akinci and Johnston 1993
	Female mice		Increase	
Swim (32°C, 3 min)	Male mice	[^{3}H]GABA, low affinity	No change	Skerritt *et al.* 1981
	Female mice		Increase	
Swim (25°C, 15 min)	Male rats	[^{3}H]Muscimol	No change	Motohashi *et al.* 1993
Swim (17°C, 10 min)	Male rats	Muscimol-stimulated Cl^- uptake	Increase	Schwartz *et al.* 1987
Foot shock	Male rats	[^{3}H]GABA, low affinity	Decrease	Biggio *et al.* 1981
	Male rats	[^{3}H]GABA, low affinity	Decrease	Cuadra and Molina 1993
	Male rat pairs	[^{3}H]GABA, low affinity	No Change	
	Male rats	[^{3}H]Muscimol	Increase	Drugan *et al.* 1993
Handling-naïve exposure to death by guillotine	Male rats	[^{3}H]GABA, low affinity	Decrease	Biggio *et al.* 1981

et al. 1981; Concas *et al.* 1985; Corda *et al.* 1985; Cuadra and Molina 1993) have been shown to affect $GABA_A$ receptor binding even in the same stress paradigm.

Binding of channel blocking agents is also affected by acute swim stress, suggesting alterations in functional GABA binding sites consistent with altered low-affinity GABA binding (Havoundjian *et al.* 1986). [^{35}S]TBPS binds within the channel domain of the $GABA_A$ receptor. Reduced binding of [^{35}S]TBPS is observed in the presence of orthosteric and allosteric agonists and enhanced binding is observed in the presence of orthosteric and allosteric site antagonists (Concas *et al.* 1987, 1988b). The authors of many studies examining [^{35}S]TBPS binding speculate that changes in binding of this radioligand reflect changes in the availability of $GABA_A$ receptor binding sites, and receptors that are bound by [^{35}S]TBPS are thought to be in a 'non-functional' antagonist-preferring conformation with reduced ability to conduct chloride ions (Concas *et al.* 1986, 1987; Havoundjian *et al.* 1986). Thus, the consistently observed increase in the number and affinity of [^{35}S]TBPS sites in the brain following various stressors such as foot shock (Concas *et al.* 1987, 1988a, 1993), exposure to carbon dioxide gas (Concas *et al.* 1993), restraint stress (McIntyre *et al.* 1988), swim stress (Havoundjian *et al.* 1986), and learned helplessness (Drugan *et al.* 1994) is thought to represent an increase in non-functional receptors and correlates with the reduced binding at the low-affinity orthosteric site observed by the same groups in separate studies.

Effects of acute stress on $GABA_A$ receptor allosteric binding sites

As was the case for agents binding to the orthosteric binding site, the effect of stress on benzodiazepine binding in rodents varies depending on the stress paradigm (see Table 2) and effects are typically of smaller magnitude than changes observed in the orthosteric site (Braestrup *et al.* 1979). For example, while male mice show no changes in benzodiazepine site binding following swim stress and isolation (Braestrup *et al.* 1979; Skerritt *et al.* 1981; Park *et al.* 1993), foot shock, and social immobilization resulted in increased and decreased benzodiazepine binding, respectively, in forebrain cortical regions (Braestrup *et al.* 1979).

Regional information is available for stress-induced changes in benzodiazepine binding, but is largely inconsistent. Some studies show increased binding at the benzodiazepine site compared with controls in the cortex (Soubrie *et al.* 1980; Rago *et al.* 1989; Motohashi *et al.* 1993) but not the hippocampus or cerebellum (Motohashi *et al.* 1993) following swim stress. On the other hand, others have consistently found decreases in the binding of both the positive allosteric modulator [^{3}H]flunitrazepam, and the negative allosteric modulator [^{3}H]-β-carboline-3-carboxylate ethyl ester (βCCE), in the cortex of male rats following swim stress (Medina *et al.* 1983a,b).

Differences in stress-induced changes in benzodiazepine binding also appear to depend on the radioligand examined as no change was observed in the binding of benzodiazepine agonists in males subject to social defeat (Miller *et al.* 1987) and swim stress (Park *et al.* 1993), but changes were observed in binding of a neutralizing modulator flumazenil ([^{3}H]Ro 15-1788) in the same mice. Thus, changes in both the number of sites and the preferred conformation of the benzodiazepine site (Miller *et al.* 1987; Park *et al.* 1993) may result from stress, but the effects are not as large or consistent as those seen for the orthosteric site.

Studies in chicks subjected to swim stress have found more consistent increases in forebrain benzodiazepine sites (Martijena *et al.* 1992; Salvatierra *et al.* 1994; Benavidez and Arce 2002). Interestingly, these increases in benzodiazepine binding appear to be explained by a rapid recruitment of the benzodiazepine receptor from a pool that is unmasked using Triton X solubilization in controls (Benavidez and Arce 2002). Furthermore, disruption of microtubules and phosphorylation prevents stress-induced increases in the benzodiazepine site (Martijena *et al.* 1992) suggesting a role for receptor trafficking in the rapid alterations of $GABA_A$ receptors following acute stress.

Sex differences in the effects of acute stress on $GABA_A$ receptors

There appear to be sex differences in the effects of stress on GABA binding to $GABA_A$ receptors. Studies have shown rapid increases in female, but no change in the number (B_{max}) of male low-affinity [^{3}H]GABA binding sites following acute swim stress (Skerritt *et al.* 1981; Akinci and Johnston 1993, 1997; Skilbeck *et al.* 2008). Interestingly, comparisons of unwashed and well-washed crude membrane preparations used for [^{3}H]GABA binding show that female mice appear to contain higher concentrations of endogenous inhibitors of [^{3}H]GABA binding compared with male mice (Akinci and Johnston 1993). Thus, in general stress appears to induce an increase in functional binding sites in females and various changes in males, apparently dependent on the stress-paradigm examined.

In contrast to the orthosteric site, sex differences in benzodiazepine site binding following stress have not been observed. In mice, only one study looked at females and found no change in [^{3}H]diazepam binding in the forebrain following warm water swim stress in males or females. This study also suggests that the large changes observed in binding at the orthosteric site in stressed females are not accompanied by changes in allosteric site binding (Skerritt *et al.* 1981) suggesting stress has greater effects on non-γ_2-containing $GABA_A$ receptor subtypes. In turn this suggests that stress rapidly alters the number of binding sites at extrasynaptic sites.

Table 2 Stress-induced changes in $GABA_A$ receptor benzodiazepine site binding (B_{max})

Stress	Animal	Radioligand	Change	Region	Reference
Swim (25°C, 15 min)	Male rats	[^{3}H]Flunitrazepam	Increase No change No change	Cortex Hippocampus Cerebellum	Motohashi *et al.* 1993
Swim (6°C, 3 min)	Male rats	[^{3}H]Flunitrazepam	Increase No change	Cortex Cerebellum	Soubrie *et al.* 1980
Swim	Male rats	[^{3}H]Flunitrazepam	Increase	Cortex	Rago *et al.* 1989
Swim (18°C, 15 min)	Rats	[^{3}H]Flunitrazepam [^{3}H]βCCE	Decrease	Cortex	Medina *et al.* 1983a,b
Swim (6°C, 10 min)	Male mice	[^{3}H]Flunitrazepam [^{3}H]Ro 15-1788	No change No change No change Decrease No change Increased	Cortex Hippocampus Cerebellum Cortex Hippocampus Cerebellum	Park *et al.* 1993
Swim (32°C, 3 min)	Male mice Female mice	[^{3}H]Diazepam	No change No change	Forebrain	Skerritt *et al.* 1981
Swim (25°C) Foot shock Immobilization Isolation	Male mice	[^{3}H]Diazepam	No change Decrease Increase No change	Forebrain	Braestrup *et al.* 1979
Handling	Male rats	[^{3}H]Flunitrazepam	Decrease	Cortex	Andrews *et al.* 1992
Social defeat	Male mice	[^{3}H]Flunitrazepam [^{3}H]Ro 15-1788	No change Increase	Cortex Hypothalamus Cerebellum Cortex Hypothalamus Cerebellum	Miller *et al.* 1987
Conflict Foot shock	Male rats	[^{3}H]Diazepam	Decrease	Cortex	Lippa *et al.* 1981
Swim (38°C, 15 min)	Male chicks Female chicks	[^{3}H]Flunitrazepam	Increase	Forebrain	Salvatierra *et al.* 1994; Benavidez and Arce 2002; Martijena *et al.* 1992; Primus and Kellogg 1991
Learned helplessness	Male rats	[^{3}H]Ro 15-1788	Decrease Decrease Decrease No Change No Change	Cortex Hippocampus Striatum Cerebellum Hypothalamus	Drugan *et al.* 1989
Foot shock	Male rats	[^{3}H]Ro 15-1788	No Change	Cortex Hippocampus Striatum Cerebellum Hypothalamus	Drugan *et al.* 1989

$GABA_A$ receptor-mediated behavioral changes following acute stress

Acute stress is associated with several physiological and behavioral effects, some of which may be related to the rapid stress-induced changes in the number of $GABA_A$ receptor binding sites. Stress is associated with enhanced analgesia and in mice it has been demonstrated that a variety of stressors result in non-opioid-dependent analgesia (Skerritt *et al.* 1981). $GABA_A$ receptors have been implicated in stress-induced analgesia as administration of picrotoxin or bicuculline suppresses the analgesic effect in mice (Tokuyama *et al.* 1992). Furthermore, neurosteroids such as allopregnanolone that are released during stress (see following section on endogenous modulators) and act as potent modulators of $GABA_A$ receptors are shown to produce analgesia when injected into the cerebral ventricles of mice (Kavaliers and Wiebe 1987). Interestingly, different types of stressors appear to recruit different GABA receptor classes to

varying extents. For example, swimming and psychological stressors in mice result in an analgesic effect that is suppressed in the presence of bicuculline, a $GABA_A$ receptor antagonist, but not phaclofen, a $GABA_B$ receptor antagonist. In contrast, foot shock stress-induced analgesia was suppressed when phaclofen was administered and to a lesser extent when bicuculline was administered (Tokuyama *et al.* 1992). Thus, the neurochemical basis for stress-induced analgesia appear to be dependent on the type of stressor in mice, consistent with the varying results between $GABA_A$ receptor binding studies using different stressors.

Acute stress is also associated with changes in learning and memory, and in rats these effects have been associated with $GABA_A$ receptors. Restraint stress enhances performance on the Morris water maze, a measure of spatial memory. In rats restraint stress over a 30-min period resulted in reduced α_1 subunit mRNA in the hippocampus and prefrontal cortex but not the striatum (Zheng *et al.* 2007). These changes in $GABA_A$ receptor α_1 subunit mRNA expression occurred over a time course that was associated with activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase cascades (Zheng *et al.* 2007) that are thought to be essential for memory consolidation in associative memory tasks (Atkins *et al.* 1998). Furthermore, administration of the $GABA_A$ receptor agonist muscimol inhibited, while administration of bicuculline improved, stress-induced enhancement of performance in the Morris water maze (Zheng *et al.* 2007). Thus, the rapid stress-induced changes in the number of functional $GABA_A$ receptors in cortical and hippocampal regions may underlie such changes in learning and memory and activation of extracellular signal-regulated kinase/mitogen-activated protein kinase pathways that are observed following acute restraint stress.

Also consistent with stress-induced changes in $GABA_A$ receptor sensitivity and binding site number are studies showing that acute stress alters behavioral sensitivities to $GABA_A$ receptor ligands. In males, forced swim stress has been observed to remove anxiolytic effects of diazepam on the dark–light exploratory behavior test (Briones-Aranda *et al.* 2005), reduce the antiseizure efficacy of benzodiazepines (Deutsch *et al.* 1990), and reduce the seizure-threshold for bicuculline and picrotoxin (Soubrie *et al.* 1980; Drugan *et al.* 1985; Abel and Berman 1993; Pericic *et al.* 2001). These findings suggest altered sensitivity of $GABA_A$ receptors to these compounds. A reduction in the sensitivity to benzodiazepines may reflect modified $GABA_A$ receptor subunit composition as the removal of the γ_2 subunit (Pritchett *et al.* 1989) or the addition of α_4 or α_6 subunits would preclude benzodiazepine binding (Wisden *et al.* 1991). However, the apparent enhancement of sensitivity to picrotoxin is consistent with the increased number of binding sites observed for this compound following stress in males. Thus, altered behavioral sensitivity to $GABA_A$ receptor ligands following stress in males may result from either, or both, variations in the subunit composition of receptors, or a switch to the 'non-functional' antagonist-preferring conformation of these receptors that is apparent in postmortem binding studies. Interestingly, the stress-induced reduction in the convulsive activity of $GABA_A$ receptor antagonists is blocked by finasteride inhibition of THDOC synthesis (Barbaccia *et al.* 1998) implicating neurosteroids in the effects of stress on behaviors mediated via $GABA_A$ receptors.

Endogenous mediators of $GABA_A$ receptors: a potential mechanism for the effects of acute stress on $GABA_A$ receptors?

Steroids that influence receptors in the brain via non-genomic mechanisms are termed neuroactive steroids and neurosteroids. Neuroactive steroids are synthesized from pregnenolone (e.g. dehydroepiandrosterone), progesterone [allopregnanolone or 3α,5α-tetrahydroprogesterone (3α,5α-THP)] or corticosterone (e.g. THDOC) in the adrenal gland (see Morrow 2007). Steroids like allopregnanolone are also synthesized from cholesterol *de novo* in the brain, and are termed neurosteroids. Neuroactive steroids and neurosteroids are released in response to stress, and potently modulate $GABA_A$ receptors (Majewska *et al.* 1986; Barker *et al.* 1987; Puia *et al.* 1990; Purdy *et al.* 1991; Lambert *et al.* 1995; Robel *et al.* 1999; Hosie *et al.* 2006; Morrow 2007; Smith *et al.* 2007). During acute stress, the increase in neurosteroids has a negative feedback effect on HPA activation, dampening corticotropin releasing factor and adrenocorticotropic hormone release to attenuate the release of corticosterone (Owens *et al.* 1992; Patchev *et al.* 1992, 1994; Morrow 2007). This effect of neurosteroids is thought to restore homeostasis in acute stress situations (Biggio *et al.* 2007).

At higher concentrations, these endogenous steroids act as direct agonists on the $GABA_A$ receptor (Cottrell *et al.* 1987). Cortisol acts as a bidirectional modulator of GABA function with enhancement at low concentrations (pM) and inhibition at higher concentrations (nM) and cortisone inhibits GABA function at low concentrations (pM) in guinea pig ileum preparations (Ong *et al.* 1987, 1990). In contrast, sulfated steroids such as pregnenolone sulfate and dehydroepiandrosterone sulfate are low potency (μM) negative modulators of $GABA_A$ receptors (Majewska and Schwartz 1987; Majewska *et al.* 1990). Steroid action at $GABA_A$ receptors is affected by phosphorylation state and subunit composition, with the δ subunit appearing most sensitive to steroid enhancement (Mihalek *et al.* 1999; Belelli *et al.* 2002; Stell *et al.* 2003; Belelli and Lambert 2005).

Following stress, neurosteroids are rapidly elevated in the brain, but not in plasma of adrenalectomized rats (Purdy *et al.* 1991). In intact (non-adrenalectomized) animals, increases in brain and plasma concentrations of neurosteroids have been observed following swim stress (Purdy *et al.*

1991; Mele *et al.* 2004), exposure to foot shock and to carbon dioxide inhalation (Barbaccia *et al.* 1996a, 2001). Progesterone and deoxycorticosterone show maximal increases in rat cortex 10 min after stress, with return to basal values by 30 and 60 min, respectively. In contrast, pregnanolone and allopregnanolone concentrations are maximally increased 30 min after stress and return to baseline 120 min later (Barbaccia *et al.* 1996a,b).

The rapid increase in neurosteroids that occurs following stress affects $GABA_A$ receptor composition. Certainly, when animals were killed 30 min after exposure to an acute stressor (2 min of CO_2 exposure), an up-regulation of the δ subunit, and an increase in tonic inhibitory currents mediated by receptors incorporating this subunit, was observed in dentate gyrus granule cells of mice, compared with unstressed controls (Maguire and Mody 2007). However, it is unknown if such changes occur soon enough to explain the effects of stress on $GABA_A$ receptor binding that are observed when the brain is prepared immediately following exposure to stress. While rapid stress-induced increases in steroids that alter $GABA_A$ receptor function may contribute to observations of rapid changes in $GABA_A$ receptor binding following stress, they are not sufficient to explain them, as altered [^{3}H]GABA binding occurs in the absence of endogenous mediators (Akinci and Johnston 1993). Thus, the effects of neurosteroids may be mediated by their effects on receptor trafficking. Such rapid changes in binding site availability may be associated with the known effects of neurosteroids on $GABA_A$ receptor phosphorylation (Brussaard and Koksma 2003).

In summary, although the effects of acute stress on $GABA_A$ receptors are complicated by differences in findings according to the stress paradigm used and the sex of the animal examined, the literature is abundant with evidence that acute stress in adulthood induces rapid changes in the number of $GABA_A$ receptor binding sites. These rapid stress-induced alterations in $GABA_A$ receptor binding sites suggest rapid changes in inhibitory tone may occur in the brain with stress, perhaps mediated via receptor trafficking or changes in endogenous GABA receptor modulatory substances.

Early-life stress and $GABA_A$ receptors

Early-life environment: impact in adulthood?

Clinical and epidemiological studies are increasingly showing a relationship between the early postnatal environment and long-term neurobiological and psychological development. While genetics is unquestionably of great importance, in humans early postnatal environmental factors can increase the risk of developing psychiatric disorders, cardiovascular disorders, adult obesity, and diabetes (Lissau and Sorensen 1994; Canetti *et al.* 1997; McCauley *et al.* 1997; Russak and Schwartz 1997; Felitti *et al.* 1998). Thus, an understanding of the long-term effects of postnatal environmental disturbances is highly relevant to a number of human diseases.

Models of interrupted early-life environment have been examined for over 50 years (see Levine 1957) leading to the development of a number of animal models to examine the effects of early-life stress on adulthood physiology and behavior. Table 3 outlines the number of models in use and the nomenclature proposed by Pryce and Feldon (2003).

The most commonly used experimental designs providing the most robust adulthood differences are comparisons of early handled (EH) and non-handled (NH) groups. Other paradigms of early-life manipulation presented in Table 3 appear similar on measures of behavior and stress reactivity to either the EH or NH groups (reviewed in Plotsky *et al.* 2005; Pryce *et al.* 2001). The EH group represents a standardized 'normal' rearing condition for laboratory rodents not achieved in the animal facility rearing (AFR) group because of variations among breeding facilities (Pryce *et al.* 2002). The NH group is left undisturbed by both experimenters and animal house staff over postnatal day (PND) 1–14.

Across studies it has been observed that differences in early-life environment result in enduring alterations to behavior and stress reactivity. For example, relative to the EH group, the NH group have a more anxious (Fernandez-Teruel *et al.* 1991; Cabib *et al.* 1993; Nunez *et al.* 1995; Vallee *et al.* 1997; D'Amato *et al.* 1998; Steimer *et al.* 1998; McIntosh *et al.* 1999; Meerlo *et al.* 1999; Ploj *et al.* 1999;

Table 3 Early-life environmental manipulation protocols in rodents (adapted from Pryce and Feldon 2003)

Classification	Protocol
Early-life handling (EH)	Experimenter removes pups from home cage, mother, and siblings for several minutes daily over early postnatal life
Non-handled (NH)	No handling, cage cleaning, etc, from experimenters or animal house staff
Maternal separation (MS)	Separation of litter from dam for at least 1 h/day over several postnatal days
Single MS	Separation of litter from mother for one 24-h period
Early-life deprivation (ED)	Separation of pups from mother and litter for more than 1 h over several postnatal days (more than normal bouts of mother leaving the nest)
Animal facility rearing (AFR)	Varies but involves normal cage cleaning

Pryce *et al.* 2001; Moles *et al.* 2004) and fearful (Caldji *et al.* 2000b; Padoin *et al.* 2001; Pryce *et al.* 2001, 2003) adulthood behavioral phenotype. The NH group also demonstrate enhanced and prolonged release of HPA axis hormones following exposure to a stressor compared with the EH group (Levine 1967; Meaney *et al.* 1989, 1996; Plotsky and Meaney 1993; Liu *et al.* 1997). Such long-lasting behavioral and physiological changes following different early-life conditions suggest that the developing nervous system is sensitive to early life stress.

Previous studies have indicated long-lasting effects of early-life environment on multiple neurotransmitter systems in adulthood (Heim and Nemeroff 2001; Arborelius and Eklund 2007), yet the GABAergic system has largely been ignored. Here, the developmental changes in $GABA_A$ receptors that may be affected by early-life stress are reviewed prior to examining the evidence for long-lasting effects of early-life stress on $GABA_A$ receptors.

$GABA_A$ receptor onset and the developmental 'switch' in $GABA_A$ receptor α subunits

Studies measuring $GABA_A$ receptor binding sites (Schlumpf *et al.* 1983; Shaw *et al.* 1991), subunit mRNA expression (MacLennan *et al.* 1991; Zhang *et al.* 1991; Laurie *et al.* 1992; Poulter *et al.* 1992, 1993) and electrophysiological responses (Kellogg and Pleger 1989) all show that $GABA_A$ receptors are abundant and functional in early brain development, appearing by 15–18 weeks gestation in human cortex (Aaltonen *et al.* 1983), fetal day 60 in the developing macaque cortex (Shaw *et al.* 1991; Hendrickson *et al.* 1994) and around gestational day 14 in rat brainstem (Schlumpf *et al.* 1983; Poulter *et al.* 1992). The subset of $GABA_A$ receptors containing the γ_2 subunit that are labeled by benzodiazepines are highly expressed early in cortical development but they decrease during development to reach adult levels by PND14 in rats (McKernan *et al.* 1991) and by birth in primates (Shaw *et al.* 1991).

All species show developmental changes in $GABA_A$ receptor subunit protein and mRNA expression. The most striking change is the decrease in α_2 subunit expression, the predominant α subunit in early development, and maturational increase in α_1 subunit expression, the predominant adult form of α subunit (Fuchs and Sieghart 1989; Sato and Neale 1989; Gambarana *et al.* 1990, 1991; Vitorica *et al.* 1990; MacLennan *et al.* 1991; McKernan *et al.* 1991; Araki *et al.* 1992; Laurie *et al.* 1992; Poulter *et al.* 1992, 1993; Zhang *et al.* 1992; Fritschy *et al.* 1994; Hendrickson *et al.* 1994; Paysan *et al.* 1994; Hornung and Fritschy 1996; Okada *et al.* 2000; Bosman *et al.* 2002; Heinen *et al.* 2004; Lopez-Tellez *et al.* 2004). As shown by the summary of the literature in Table 4, the switch from α_2 to α_1 subunit dominance is regionally dependent, being most evident in areas such as the thalamus and lower cortical layers of primary sensory cortices (Fritschy *et al.* 1994; Hornung and Fritschy 1996), but almost non-existent in regions which maintain high α_2 expression throughout maturation such as the granule cell layer of the hippocampus, striatum and outer cortical layers (Fritschy *et al.* 1994; Hornung and Fritschy 1996) (see Table 4).

The α subunit 'switch' shows species variations in its time course. Immunoreactivity for the α_1 subunit is mostly absent from the fetal brain of humans (Reichelt *et al.* 1991; Brooks-Kayal and Pritchett 1993; Kanaumi *et al.* 2006), non-human primates (Hendrickson *et al.* 1994; Hornung and Fritschy 1996) and rodents (McKernan *et al.* 1991; Fritschy *et al.* 1994; Lopez-Tellez *et al.* 2004) while α_2 immunoreactivity is prominent and widespread prenatally (McKernan *et al.* 1991; Fritschy *et al.* 1994; Hornung and Fritschy 1996; Lopez-Tellez *et al.* 2004). $\alpha_{2/3}$ Subunit mRNAs are first detected in rat brain at embryonic day (ED) 15 with α_5 appearing at ED 17. α_1 Subunit mRNA appears in the rat cortex at ED 19 and PND5 in the hippocampus (Poulter *et al.* 1992; Lopez-Tellez *et al.* 2004). Immunoreactivity for the α_1 subunit is first seen in regions of the brainstem, cerebellum, basal forebrain, and primary sensory cortices (layer III–IV and VI of visual and somatosensory; SS) during the last weeks of gestation in primates, or just after birth in rodents (McKernan *et al.* 1991; Fritschy *et al.* 1994; Paysan *et al.* 1994; Lopez-Tellez *et al.* 2004). The adult α subunit immunoreactivity pattern is generally observed by the onset of behavioral and sexual maturity for rats (21 days) (Fritschy *et al.* 1994) and marmosets (3 years) (Hornung and Fritschy 1996). Thus, the gradual replacement of α_2 subunits with the α_1 subunit occurs largely over the first two postnatal weeks in rodents and so it is highly feasible that early-life environmental manipulations over this time period may disrupt this developmental process.

Effects of early-life stress on $GABA_A$ receptors

Despite the evidence of early-life stress-induced disruptions to anxiety behaviors in adulthood and the pivotal role of $GABA_A$ receptors in anxiety, few studies have examined long-lasting changes in $GABA_A$ receptor expression or function following early-life stress. Previous studies have shown that adult rats exposed to the NH or maternal separation (MS) conditions display decreased numbers of high affinity (30 nM) [^{3}H]GABA binding sites in the medial prefrontal cortex, nucleus tractus solitarus, and locus coeruleus (Caldji *et al.* 2000a,b) as well as decreased numbers of forebrain and amygdala benzodiazepine sites (measured using [^{3}H]flunitrazepam), compared with EH animals (Bodnoff *et al.* 1987; Bolden *et al.* 1990). Consistent with early-life stress inducing long-term decreases in benzodiazepine receptors, are observations of decreased γ_2 subunit mRNA expression in the amygdala, nucleus tractus solitarus, and locus coeruleus in NH and MS groups relative to EH controls (Caldji *et al.* 2000a,b). Thus, it appears that early-life stress results in long-term decreases in benzodiazepine receptor

Table 4 Maturational changes in α_1 and α_2 subunit protein expression by region

Region		α_1	α_2	Species	Reference
Cortex	Whole	↑	↓	Rat	McKernan *et al.* 1991
	Primary sensory (BA17, S1)	↑	↓	Marmoset, rat, macaque	Hornung and Fritschy 1996; Fritschy *et al.* 1994; [a]Paysan *et al.* 1994; Hendrickson *et al.* 1994
	Temporal	↑[b]	0	Human	Kanaumi *et al.* 2006
	Motor, association areas	↑	↓	Marmoset, rat, macaque	Hornung and Fritschy 1996; Fritschy *et al.* 1994; Paysan *et al.* 1994; Hendrickson *et al.* 1994
	Infragranular layers	↑[b]	0	Marmoset, rat, macaque	Hornung and Fritschy 1996; Fritschy *et al.* 1994; Paysan *et al.* 1994; Hendrickson *et al.* 1994
Hippocampus	Whole	↑	0	Human, marmoset, rat	Kanaumi *et al.*2006; Hornung and Fritschy 1996; Fritschy *et al.* 1994; Lopez-Tellez *et al.* 2004
	Dentate gyrus	↑[b]	↓	Human, rat	Kanaumi *et al.* 2006; Lopez-Tellez *et al.* 2004
	CA1	↑	↑	Human, rat	Kanaumi *et al.* 2006; Davis *et al.* 2000; Lopez-Tellez *et al.* 2004
	CA3	0 (Human) ↑ (Rat)	0	Human, rat	Kanaumi *et al.* 2006; Davis *et al.* 2000; Lopez-Tellez *et al.* 2004
Thalamus	Whole	↑	↓	Marmoset, rat	Hornung and Fritschy 1996; Fritschy *et al.* 1994
	Ventrolateral nucleus	↑	↓	Rat	Davis *et al.* 2000
	Laterodorsal nucleus	↑	↓	Rat	Okada *et al.* 2000
Hypothalamus	Pre-optic area	0	↑	Rat	Davis *et al.* 2000;
	Ventromedial nucleus	↓	↑		
Amygdala	Whole	↓	↓	Rat	Davis *et al.* 2000
Basal Forebrain	Globus pallidus	↑	↓	Marmoset, rat	Hornung and Fritschy 1996; Fritschy *et al.* 1994
	Substantia nigra	↑	↓		
	Medial septum	↑	↓		
	Pallidum	↑	↓		
Cerebellum	Whole	↑	↓	Rat	McKernan *et al.* 1991; Fritschy *et al.* 1994
Brainstem	Pre-botzinger complex	↑	0	Rat	Liu and Wong-Riley 2004
	NTS	↑		Rat	Liu and Wong-Riley 2006
	Cuneate	↑			

[a]Denotes references that only apply to α_1 subunit changes, [b]denotes transient change. NTS, nucleus tractus solitarus.

binding, with the amygdala being identified as a forebrain region relevant to such changes.

Early-life stress also appears to influence the $\alpha_{1/2}$ subunit 'switch' that occurs in rodents during the early postnatal period, suggesting environmental manipulations may affect GABAergic system development. Hippocampal dentate gyrus cells from adult rats given two handling separations (30 min/6 h; MS) before PND10 were less sensitive to zolpidem enhancement of GABAergic currents and showed longer current decay times relative to AFR controls (Hsu *et al.* 2003), indicative of a reduced α_1 subunit contribution. These findings were confirmed by observations of decreased α_1 and increased α_2 subunit mRNA without evidence of cell loss in the dentate gyrus of the MS group relative to the AFR controls (Hsu *et al.* 2003).

Surprisingly, no previous studies have examined changes in α subunit expression in regions such as the primary sensory cortices and the thalamus, where the developmental subunit switch in α subunits is most prominent. However, recent work in our laboratory has suggested that early-rearing environment has regionally dependent effects on adult $GABA_A$ α_1 and α_2 subunit immunoreactivity. In adulthood, NH males showed reduced α_2 subunit protein expression in layers I, IV, V, and VI of the SS cortex and the lateral–dorsal thalamic nucleus relative to EH males. Similarly, adult NH females showed a reduction in α_2 subunit protein density in the lower SS cortical layers as well as an increase in α_1 subunit density in the lower layers (IV, V, and VI) of the cortex (unpublished observations). Interestingly, both males and females exposed to the NH early-life stress condition showed altered $\alpha_1 : \alpha_2$ subunit ratios in brain regions where the α subunit switch is prominent (unpublished observations). Thus, adulthood effects of early-life stress on $GABA_A$ receptors may arise because of disruptions of the developmental processes governing $GABA_A$ receptor maturation.

In summary, the effects of early-life intervention protocols on $GABA_A$ receptors indicate that early-life stress produces long-lasting changes in benzodiazepine binding site number and alterations in $GABA_A$ receptor subunit expression. Alterations in α_1 and α_2 subunit expression that arise from differences in early-rearing environment suggest that early-

life environment may induce long-term changes in behavior and stress reactivity via disruptions in developmental processes such as the $GABA_A$ receptor $\alpha_1 : \alpha_2$ subunit switch that occurs over the early postnatal period. Given that the α_1 and α_2 subunits are thought to be responsible for mediating different behaviors via $GABA_A$ receptors (Brooks-Kayal and Pritchett 1993; Kapur and MacDonald 1999; Low *et al.* 2000; Okada *et al.* 2000; Juttner *et al.* 2001; Bosman *et al.* 2002; Reynolds *et al.* 2003), disruptions in the developmental α subunit 'switch' may provide a molecular basis for the effects of early-life stress on adulthood anxiety.

Steroid regulated $GABA_A$ receptor plasticity: effects on stress responsivity across the lifespan?

Unlike the permanent switch from α_2 to α_1 subunit predominance that occurs in the early development of the brain, recent work has shown $GABA_A$ receptors containing δ subunits demonstrate a fluid expression throughout their lifespan. $GABA_A$ receptors containing the α_4 and δ subunits are more sensitive to neurosteroids than other subtypes and appear to be expressed in a fashion that is regulated by these steroids (Shen *et al.* 2005). In females, variations in neurosteroid metabolites of progesterone during the ovarian cycle and pregnancy lead to transient changes in the expression of δ subunits. For example, when progesterone is elevated during pregnancy, increased δ and decreased γ_2 subunit protein is observed in various regions of the hippocampus (Sanna *et al.*, 2009; Maguire and Mody 2008), thalamus, and striatum (Maguire *et al.* 2009), while reduced γ_2 subunit mRNA is observed in the rat cortex (Follesa *et al.* 1998; Concas *et al.* 1999). Similarly, during the ovarian cycle when progesterone levels are elevated, δ subunit protein is up-regulated in the hippocampus (Maguire *et al.* 2005) and the periaqueductal gray (Griffiths and Lovick 2005; Lovick *et al.* 2005). Finasteride treatment, which blocks steroid synthesis, prevents the $GABA_A$ receptor δ subunit up-regulation and corresponding increases in tonic inhibitory currents that are observed during pregnancy (Concas *et al.* 1999; Sanna *et al.*, 2009), and the ovarian cycle (Maguire and Mody 2008). Together these findings suggest that at least for females, fluctuating neurosteroid levels may affect the baseline GABAergic tone, which in turn may alter the effects of stress on the brain, and account for sex differences in the effects of acute stress on the brain.

Recent evidence has shown that neurosteroids also induce a transient developmental switch in the expression of $GABA_A$ receptors. In mice of both sexes, the expression of $\alpha_4\beta\delta$ $GABA_A$ receptor protein on CA1 hippocampal pyramidal cells increases at the onset of puberty following a prolonged elevation of allopregnanolone prior to puberty (Shen *et al.* 2007). Interestingly, $\alpha_4\beta_2\delta$ receptors show an outward chloride current at this time (Shen *et al.* 2007). Under these conditions, the neurosteroid allopregnanolone acts to reduce the tonic inhibition, leading to an increase in activity of CA1 pyramidal cells and corresponding increases in anxiety following administration of allopregnanolone (Shen *et al.* 2007). By adulthood, $\alpha_4\beta_2\delta$ receptors are still expressed at high levels on the dendrites of granule cells in the dentate gyrus of the hippocampus, but outward chloride currents, and the pro-anxiety effects of allopregnanolone, are no longer observed (Shen *et al.* 2007). Such anxiety promoting effects of allopregnanolone at the onset of puberty might contribute to the aversive effects of stress that emerge at puberty in humans (Shen *et al.* 2007). Furthermore, given the rapid increases in the concentration of neurosteroids in the brain that are observed with stress (Purdy *et al.* 1991; and see previous section on early-life stress), it is expected that the effects of stress on $GABA_A$ receptors at puberty may vary to those observed in adulthood.

In summary, this new evidence regarding the effects of neurosteroids and changes in subunit expression at key periods of development such as during puberty are crucial when considering the effects of stress and age of exposure to stress. Together, these studies show that neurosteroids are crucial to plasticity in $GABA_A$ receptors containing the δ subunit and via affecting the expression of this subunit, neurosteroids may alter GABAergic tone at various points during the lifespan. Furthermore, the effects of endogenous neurosteroids released during stress may be reversed at puberty suggesting that both the effects of stress on the brain, and the behavioral responses to stress, will vary over the lifetime.

Conclusions

The findings reviewed here indicate that $GABA_A$ receptors are sensitive to subtle changes in the environment in both early-life and adulthood. The stress sensitivity of $GABA_A$ receptors both in the short- and long-term suggests that both behaviors and clinically relevant drugs that are mediated via this system may be affected by prior stressful experiences throughout the lifespan. Further investigation into the roles of neurosteroids, phosphorylation, and receptor trafficking in mediating stress-induced changes in $GABA_A$ receptors may help our understanding of the mechanism by which $GABA_A$ receptors are affected by stress in the short- and long-term. Furthermore, given the diathesis-stress models of many human diseases, in which adulthood stress is hypothesized to precipitate the expression of disease symptoms in predisposed individuals, it will be interesting to ascertain if adulthood stress reactivity in the GABAergic system is affected following early-life stress.

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