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## Neuropeptide-mediated excitability: a key triggering mechanism for seizure generation in the developing brain

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### Abstract

Most human seizures occur early in life, consistent with established excitability-promoting features of the developing brain. Surprisingly, the majority of developmental seizures are not spontaneous but are provoked by injurious or stressful stimuli. What mechanisms mediate ‘triggering’ of seizures and limit such reactive seizures to early postnatal life? Recent evidence implicates the excitatory neuropeptide, corticotropin-releasing hormone (CRH). Stress activates expression of the CRH gene in several limbic regions, and CRH-expressing neurons are strategically localized in the immature rat hippocampus, in which this neuropeptide increases the excitability of pyramidal cells *in vitro*. Indeed, *in vivo*, activation of CRH receptors – maximally expressed in hippocampus and amygdala during the developmental period which is characterized by peak susceptibility to ‘provoked’ convulsions – induces severe, age-dependent seizures. Thus, converging data indicate that activation of expression of CRH constitutes an important mechanism for generating developmentally regulated, triggered seizures, with considerable clinical relevance.

The majority of seizures occurring in the developing human are not spontaneous, that is, they are not related to inherent abnormalities in the balance of neuronal excitation and inhibition<sup>1</sup>. Rather, most seizures during infancy and childhood are provoked or triggered by alterations in the normal milieu of excitable neurons<sup>2</sup>. Thus, rapid triggering by fever, hypoxia or trauma provokes the majority of seizures in the infant and young child. These seizures – a manifestation of rapid and transient enhancement of neuronal excitability in response to adverse and potentially injurious agents – demonstrate an exquisite age specificity (Table 1): febrile seizures are exclusive to infancy and early childhood<sup>1,2</sup>, immediate traumatic seizures (as opposed to posttraumatic epilepsy) are far more common in the young human compared with the adult, and anoxia-related seizures occur primarily in the full-term neonate<sup>5,7</sup>. Other seizures that are not genetic in origin, such as infantile spasms, which have been linked to a large number of injuries of the CNS and stressors that include infections and malformations, are highly age specific and primarily restricted to the first year of life<sup>6</sup>.

In the present review, we focus on novel and evolving concepts regarding potential mechanisms for the rapid transduction of stressful alterations of neuronal milieu into enhanced excitability and resulting seizures. We discuss the cascade of molecular events triggered by ‘stress’, with an emphasis on the excitatory neuropeptide, corticotropin-releasing hormone (CRH). Established and novel *in vivo* and *in vitro* evidence for the role of CRH in enhancing excitation in key limbic circuits, particularly in the tri-synaptic hippocampal pathway, are discussed. Finally, we describe recent data regarding the receptors that mediate CRH-induced excitation, and provide the probable mechanisms underlying the remarkable age specificity of the proconvulsant effects of this neuropeptide. We conclude that CRH is a likely candidate for modulating excitability in response to triggers such as fever, hypoxia or trauma, which result directly in developmentally regulated, clinically important seizures.

## The dilemma: the immature brain is more excitable – but spontaneous seizures, in the absence of prior injury, are uncommon

It is generally considered that the immature brain is more excitable than the adult brain (see Ref. 8 for a recent review). This concept is supported in the human by the much greater incidence of seizures in the infant and young child, as compared with the adult<sup>1,2,9</sup>. Enhanced excitability and a higher sensitivity to proconvulsant agents have also been demonstrated in immature experimental animals, including rats, cats and monkeys<sup>10,11</sup>. Several characteristics of developing neuronal circuitry might account for this enhanced excitability<sup>8,12</sup>. For example, GABA, the principal inhibitory neurotransmitter in the mature CNS, has depolarizing, and thus excitatory, properties during the first postnatal week in the rat<sup>12,13</sup>. In addition, excitatory amino acid receptors are both more abundant in the immature brain<sup>14</sup> and possess a subunit makeup that promotes neuronal depolarization<sup>15</sup>. These factors are thought to contribute to an altered excitation–inhibition balance during development<sup>8</sup>. At the circuit level, increased neuronal excitability is evident, leading, for example, to the robust long-term potentiation observed during the second postnatal week ('infancy' in the rat) in both hippocampal and cortical synapses<sup>16,17</sup>. In addition, the number of cortical and hippocampal excitatory synapses is highest during this period and decreases after the third post-natal week<sup>18</sup>. All of these factors favor enhanced excitation and a propensity to generation and propagation of seizures<sup>8</sup>.

Although possessing enhanced intrinsic excitability and sensitivity to proconvulsant agents, the immature brain is primarily engaged in normal neuronal functions. Indeed, the vast majority of immature humans and rodents do not generate spontaneous seizures (although experimental or other developmental injury can lead to reactive epilepsy at this age). Surprisingly, even genetically determined spontaneous seizures (genetic epilepsies) typically begin later than the 'excitable' developmental period<sup>19,20</sup>. During the neonatal and infancy periods in the human, seizures are most commonly 'triggered', that is, they are induced by acute and adverse alterations of the 'normal' neuronal milieu<sup>21</sup>. Thus, stressful circumstances, such as trauma, anoxia, fever or hypoglycemia, lead rapidly (within minutes) to enhanced excitability and seizures. Animal models of febrile convulsions<sup>3</sup> and anoxic seizures<sup>5</sup> document the restricted period of susceptibility to these seizures in the rat, which is confined to the second postnatal week<sup>3,22,23</sup>.

In summary, the immature brain is prone to seizures<sup>8</sup>, yet generation of seizures is selective: spontaneous intrinsic and genetic seizures are relatively uncommon, while rapidly triggered seizures can be provoked by diverse stressful signals.

## Stressful signals activate the excitatory neuropeptide CRH

Candidate mechanisms for the rapid stress-induced excitation of limbic neurons emerge from analyses of the molecular events induced by stress (Fig. 1). It has been well established that the CNS responds to stressful circumstances by rapid hormonal, autonomic and behavioral alterations, designed to enable survival in potentially adverse situations and restore a steady state<sup>24,25</sup>. The major mediator of the hormonal and behavioral responses to stress is the neuropeptide CRH (Refs 26,27). Within seconds of exposure to stress, CRH, located in peptidergic neurons in the paraventricular hypothalamic nucleus (PVN), is secreted from nerve terminals to influence rapid hormonal secretion from corticotrophs in the anterior pituitary<sup>28,29</sup> (Fig. 1A). In addition, stress upregulates transcription of the CRH gene in the PVN within seconds<sup>30,31</sup> (Fig. 2), leading to increased steady-state expression of CRH-encoding mRNA (Refs 29,32).

It has increasingly been recognized, however, that the actions of CRH as a neuromodulator of stress are not confined to the neuroendocrine, hypothalamic–pituitary–adrenal system (Fig. 1). Behavioral studies have demonstrated activation and arousal induced by this peptide in a number of species<sup>27,33</sup>. These CRH-induced behaviors are indistinguishable from behaviors observed under stressful circumstances<sup>34</sup>. Furthermore, administration of CRH-receptor antagonists can abolish typical stress-induced behaviors<sup>35,36</sup>. Interestingly, this blocking effect is demonstrable when the antagonists are injected into the amygdala, suggesting that the site of stress-induced CRH-receptor activation lies at least partially within this limbic region<sup>37</sup> (Fig. 1B).

Studies of the distribution of CRH in the adult and developing CNS have demonstrated the presence of significant populations of CRH-expressing neurons in discrete limbic regions<sup>38,39</sup>. In particular, a large cluster of CRH-expressing neurons is located in the central nucleus of the amygdala<sup>38,39</sup>. These cells are rapidly activated by stress to express immediate–early genes<sup>40</sup> and CRH-encoding mRNA (Refs 41,42). Stress-induced release of CRH from these amygdala neurons is evident from microdialysis data and from local infusion of competitive CRH-receptor antagonists<sup>37,43</sup>. CRH interacts with specific postsynaptic G-protein-coupled receptors<sup>44–46</sup> to alter intracellular cAMP levels.

Scattered CRH-immunoreactive neurons have also been localized to the hippocampal formation of the adult rat<sup>38,47,48</sup>. Interestingly, recent detailed double-labeling and ultrastructural studies in the hippocampal formation of the immature (10–13-day old), unstressed rat have demonstrated a robust population of CRH-expressing GABAergic interneurons<sup>49</sup>. Specifically, numerous, large CRH-immunoreactive neurons are found in CA3 strata pyramidale and oriens, and fewer in the corresponding layers of CA1 and stratum lacunosum-moleculare of Ammon's horn. In the dentate gyrus, CRH-immunoreactive cell bodies are confined to the granule cell layer and hilus. Ultrastructurally, CRH-expressing neurons have aspiny dendrites and their axon terminals form axosomatic and axodendritic symmetric synapses with pyramidal and granule cells. Other CRH-immunoreactive terminals were found to synapse on axon initial segments of principal neurons. The vast majority of CRH-expressing neurons are co-immunolabeled for the GABA-synthesizing enzyme isoforms GAD-65 and GAD-67 and most also contain parvalbumin, but none were labeled for calbindin (Fig. 3). These findings establish the presence of a significant, heterogeneous population of hippocampal CRH-immunoreactive GABAergic interneurons. Furthermore, a subpopulation of neurons that express both CRH and parvalbumin, located within and adjacent to the principal cell layers, consists of basket and chandelier cells. Thus, axon terminals of these CRH-expressing interneurons are strategically positioned to influence the excitability of the principal hippocampal neurons via release of both CRH and GABA (Ref. 49).

In summary, the neuropeptide CRH functions within the hypothalamus to regulate rapid hormonal responses to stress. However, the precise and selective distribution of CRH neurons within the amygdala and hippocampus allows rapid release of this peptide to affect the excitability of principal neurons and neuronal circuits within these key limbic regions.

## What are the neurophysiological effects of CRH?

The actions of CRH on neurons have been investigated both *in vivo* and *in vitro*, in neuronal populations as diverse as the locus coeruleus and cerebellum. For the purposes of this review, we focus on limbic neurons and discuss *in vivo* and *in vitro* studies in the mature and the developing rat.

In the adult rat *in vivo*, Ehlers *et al.* described long-latency (h) seizures induced by CRH, with onset in the amygdala<sup>50</sup>, whereas Marrosu *et al.* recorded epileptiform discharges in the

hippocampus after administration of CRH (Ref. 51). The long latency and behavioral characteristics of CRH-provoked seizures in adult rats suggested that CRH might lower the seizure threshold or act otherwise as a 'kindling stimulus' for the development of limbic seizures<sup>52,53</sup>. Weiss *et al.* demonstrated that preadministration of CRH significantly accelerated the development of stage 3 kindled seizures in mature male rats<sup>53</sup>. The duration of after-discharge throughout the kindling process was significantly longer in the CRH-pretreated animals. The data were interpreted to suggest a role for endogenous CRH in limbic excitability and a mechanistic interaction with the kindling process.

In the neonatal and infant rat (first and second postnatal weeks, respectively), administration of CRH into the cerebral ventricles leads to seizures within minutes<sup>54,55</sup>. The seizures, best characterized on postnatal days 10–13, resemble other limbic seizures behaviorally<sup>56,57</sup>. Using multiple bipolar depth electrodes, the earliest recorded epileptiform discharges produced by CRH are detected in the amygdala<sup>55</sup> and propagate to the hippocampus<sup>58</sup>. Both the electrophysiological and the behavioral seizures provoked by CRH in the developing rat persist for several hours<sup>59,60</sup>, and require doses ( $7.5 \times 10^{-12}$  mol) that are 200 times smaller than those required for generation of seizures in adults. Significantly, these doses of CRH, by themselves, do not elevate the concentration of plasma corticosterone<sup>54</sup>, excluding the possibility that glucocorticoids are involved in the initiation of CRH-produced seizures. This is particularly important in view of the established effects of corticosterone, acting via glucocorticoid-receptor activation, on  $Ca^{2+}$  currents in hippocampal CA1 pyramidal neurons<sup>61</sup>. However, CRH-induced and other seizures constitute a stressful event, leading to marked elevations of plasma corticosterone<sup>54</sup>, so that a later potentiation of hippocampal-neuron excitability by glucocorticoids cannot be excluded. In addition, *in vivo* studies in the immature rat indicate that, unlike in the adult, activation or blocking of CRH receptors does not alter any measure of the acquisition or maintenance of kindling<sup>62,63</sup>.

### CRH promotes excitability in hippocampal circuitry

Clearer insight into the effects of CRH on neuronal excitability has emerged from *in vitro* studies. Intracellular electrophysiological recordings from hippocampal CA1 and CA3 pyramidal neurons of adult rat revealed that CRH increased the firing of CA1 pyramidal neurons in response to excitatory input, and reduced afterhyperpolarization following an action-potential train elicited by depolarizing current<sup>64</sup>. Similar findings were obtained in the basolateral nucleus of the amygdala<sup>65</sup>.

In the immature rat (10–13 days old), field recordings demonstrated increased amplitudes of population spikes elicited in CA1 after administration of CRH (Refs 66,67). These changes were reversed with peptide wash-out and were prevented by preapplication of a CRH-receptor blocker. Because of the high expression of CRH receptors in the CA3 pyramidal cell layer<sup>68</sup>, whole-cell patch-clamp recordings were performed on hippocampal CA3 neurons. These studies (confirming the findings of Aldenhoff *et al.*<sup>64</sup> in the adult) demonstrated that CRH increased significantly the number of spikes elicited by depolarizing current pulses. Thus, these findings indicate that, in the presence of a strong depolarizing influence and action potential firing in the network, CRH can enhance excitability by increasing the number of output action potentials of a given cell. CRH increased the frequency of spontaneous excitatory postsynaptic currents (EPSCs) by an average of 252% (Fig. 4A), but did not alter the amplitude or kinetic properties of miniature EPSCs, indicating a lack of a direct postsynaptic effect of the peptide on AMPA-type glutamate receptors. Similarly, CRH did not alter the amplitude of the pharmacologically isolated, evoked monosynaptic glutamatergic currents. However, when the mossy fiber-evoked responses were recorded from CA3 pyramidal cells in current-clamp mode in the presence of bicuculline (Fig. 4B), CRH caused the appearance of large bursts of action potentials at

variable latency. The bursts of action potentials appeared to be elicited by longer latency and most likely polysynaptic EPSPs, in agreement with the observation that CRH increases the firing of pyramidal cells elicited by a given excitatory input<sup>67</sup>.

Taken together, these data suggest that a given excitatory stimulus in the network can be amplified by CRH, causing hyperexcitability, while in the absence of any other depolarizing input, CRH does not lead to epileptiform neuronal activity (and seizures). However, the peptide markedly enhances excitatory input (or spontaneous discharges) of CA3 pyramidal neurons, with a robust increase in the numbers of spikes, consistent with hyperexcitability. It should be noted, however, that the postsynaptic effects of synthetic CRH applied in the hippocampal slice preparation might not fully represent the activation of CRH-expressing interneurons. The latter is expected to generate the release of both endogenous CRH and GABA, leading to a potentially different net influence on principal hippocampal cells. Indeed, the neuroanatomical data showing CRH-immunoreactive interneurons synapsing on the somata and axon initial segments of principal hippocampal neurons, combined with the electrophysiological data discussed above, provide compelling evidence for the ability of CRH to create an excitable state in the immature hippocampal circuit.

Support for the ability of CRH to enhance the excitability of the immature CNS *in vivo* is provided by the finding that repeated infusions of CRH leads to a striking augmentation of the convulsant potency of subsequently administered kainic acid<sup>69</sup>. A threshold kainic acid dose, which resulted only in short bouts of automatisms in control rats, produced status epilepticus in rats pretreated with CRH four times over two days. Other investigators have shown that repeated amygdaloid seizures result in prolonged upregulation of expression of the CRH gene in GABAergic interneurons in the dentate gyrus<sup>70</sup>. Thus, CRH-mediated neuro-transmission might promote excitability and lead to limbic seizures, which, in turn, increase the level of CRH at critical hippocampal synapses, further enhancing excitability in a positive-feedback manner.

In conclusion, converging *in vitro* and *in vivo* data indicate that CRH might induce a neuronal excitable state. In the presence of CRH-receptor activation, mild glutamatergic input is amplified to result in epileptic discharges and, potentially, in seizures. It has been established that CRH is secreted rapidly after stress in the hypothalamus and the amygdala, and ongoing studies are focused on determining the effect of specific stressors on release of CRH from hippocampal terminals. Thus, currently available data support a scenario in which select stressful circumstances lead to rapid release of CRH, which transforms normal glutamatergic excitatory input to amygdala and hippocampal neurons into epileptic output.

### **What are the postsynaptic mechanisms mediating CRH-induced excitability? Why are the peptide's effects most prominent in the developing brain?**

Specific G-protein-coupled receptors for CRH have been mapped to the adult<sup>46,71</sup> and immature rat limbic system<sup>68,72</sup>. Of the two characterized members of the CRH-receptor family – CRF<sub>1</sub> and CRF<sub>2</sub> – selective non-peptide antagonists indicate that CRF<sub>1</sub> activation is required for the excitatory effects of the neuropeptide<sup>60</sup>. Both binding studies and receptor type-specific *in situ* hybridization have shown that CRF<sub>1</sub>-receptor levels peak in the hippocampus and the amygdala during development (postnatal days 6 and 9, respectively), at 400–600% of adult levels<sup>68,73</sup>. The increased abundance of CRH receptors might partially explain the potent proconvulsant effects of this peptide during the first weeks of life. Rapid Ca<sup>2+</sup> influx after application of CRH has been demonstrated in a variety of cell types, including astrocytes, corticotrophs and neurons<sup>74–76</sup>. Administration of CRH has been



shown to injure immature hippocampal CA3 and amygdala neurons<sup>77,78</sup>. In addition, recent evidence points to a role for endogenous CRH in hippocampal neuronal death after glutamate-receptor activation, ischemia or status epilepticus in the adult<sup>79–81</sup>, perhaps contributing to the induction of pro-epileptic changes in neuronal excitability<sup>82</sup>. Taken together, these data indicate that under circumstances leading to enhanced availability of CRH at abundant CRF<sub>1</sub>-receptor sites in the immature hippocampus, the neuropeptide not only enhances excitation but potentiates excitotoxicity in selected limbic circuits.

## Conclusions: the significance of CRH to developmental seizures in the human

The present review has focused on the potential role of CRH, a stress neurohormone, as the key neuromodulator of triggered excitability in the developing brain. Many features of the immature brain promote excitability, but the relative paucity of spontaneous seizures suggests that a balance of excitation and inhibition exists during this period under steady-state conditions. Perturbation of this balance by select injurious or stressful signals might lead to seizures, via enhanced secretion of CRH, which acts on specific receptors in the hippocampus and amygdala to promote excitability. CRH receptors in these regions are maximally expressed during the time of peak CRH-induced excitability and of propensity to triggered seizures, suggesting that regulation of expression of CRH receptors might play a key role in modulating the ability of extrinsic signals to provoke limbic seizures. In human infants, certain age-specific seizures respond to ACTH, a downregulator of CRH synthesis<sup>83,84</sup>, and CRH-receptor antagonists increase seizure threshold in an animal model of febrile seizures, the most common age-specific seizures of the developing human<sup>85,86</sup>. These data further support the notion that selective blockers of CRH-receptor activation might be useful anticonvulsants for age-specific, provoked seizures in the developing human.

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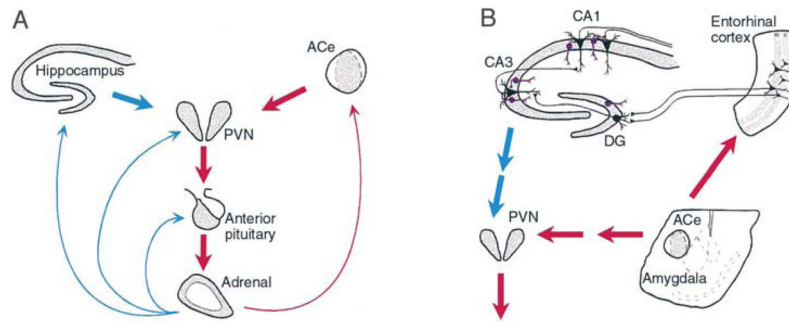
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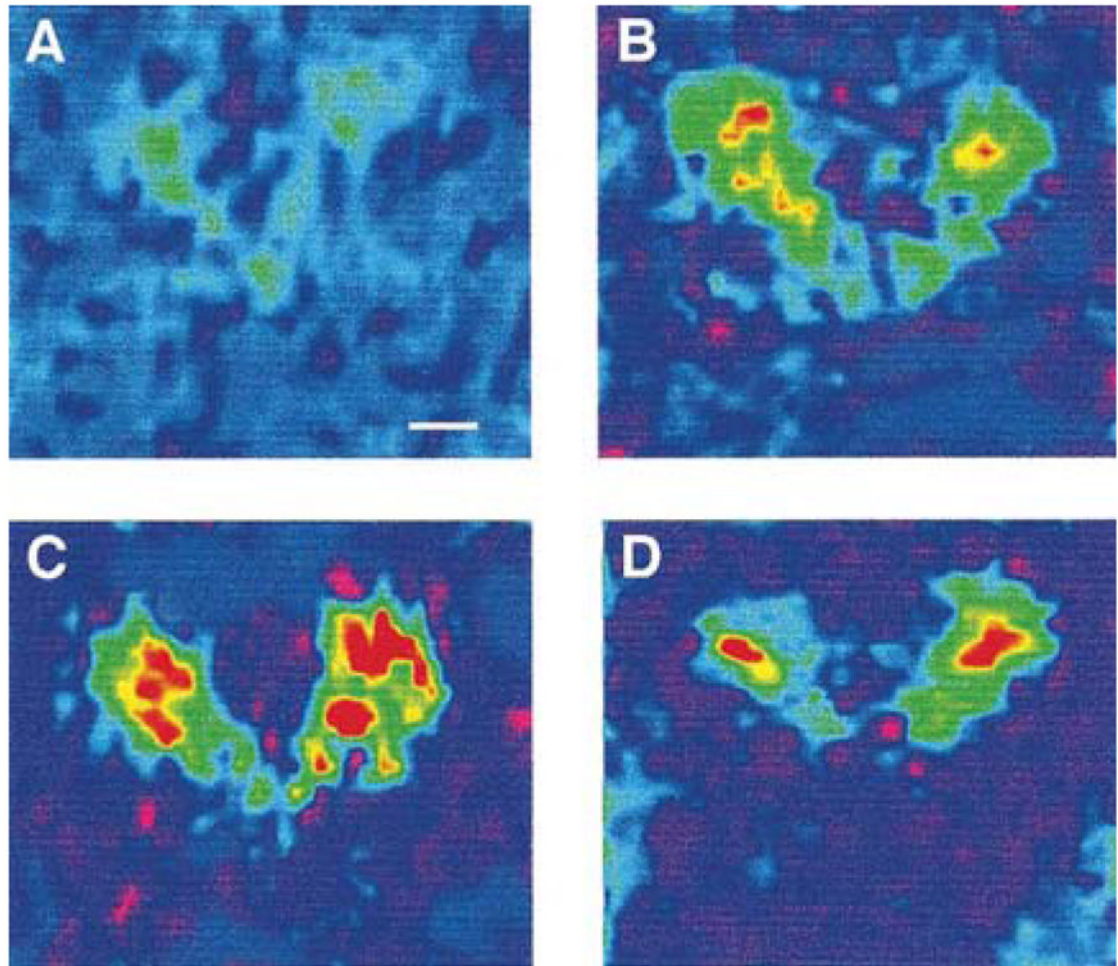
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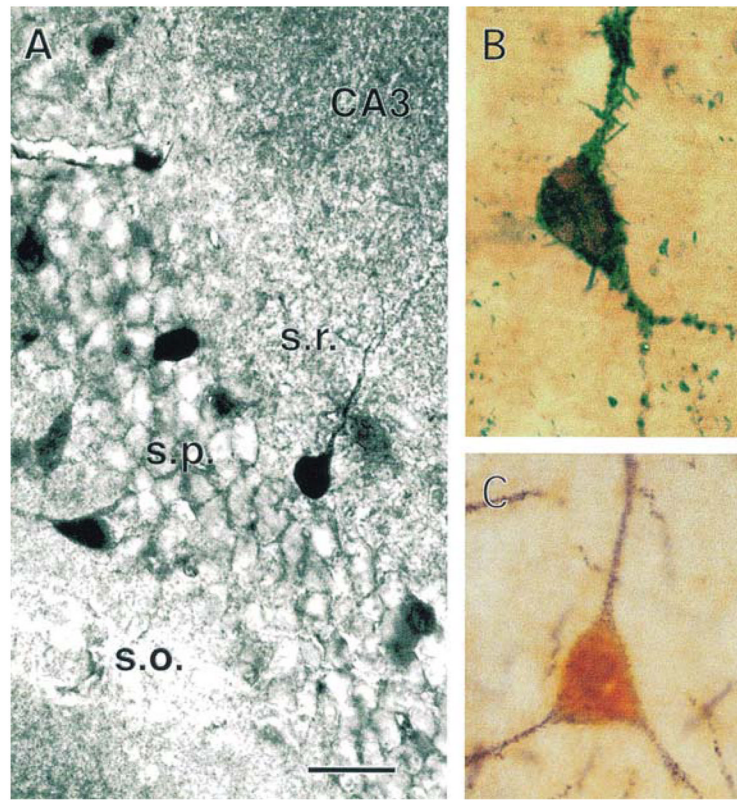
**Fig. 1. The neuroendocrine (A) and limbic (B) interactive, stress-activated corticotropin-releasing hormone (CRH) loops**

(A) Stress-conveying signals rapidly activate immediate-early genes in CRH-expressing neurons of the central nucleus of the amygdala (ACe). Rapid CRH release in the ACe is associated with CRH-expression in hypothalamic paraventricular nucleus (PVN) and secretion of CRH into the hypothalamo-pituitary portal system, inducing ACTH and glucocorticoid secretion from the pituitary and adrenal, respectively. Glucocorticoids exert a negative feedback on the PVN (directly and via the hippocampus), yet activate expression of the CRH gene in the amygdala, potentially promoting further CRH release in this region. (B) CRH-expressing GABAergic interneurons (purple cells) in the principal cell layers of the hippocampal CA1, CA3 and the dentate gyrus (DG) are positioned to control excitability of the pyramidal and granule cells, respectively. These neurons might be influenced by stress-evoked release of CRH from the ACe, via connections in the entorhinal cortex. For both panels, red and blue arrows denote established or putative potentiating and inhibitory actions, respectively. Arrows do not imply monosynaptic connections.



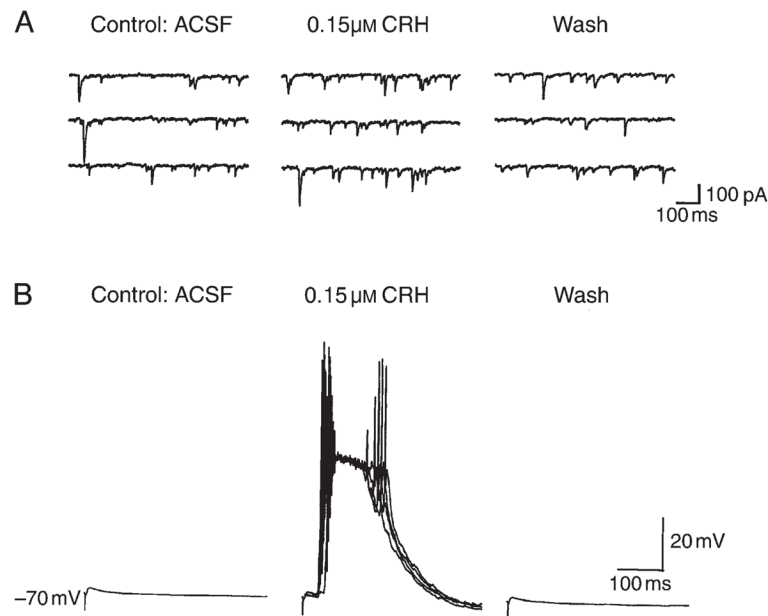
**Fig. 2. Rapid activation of expression of the corticotropin-releasing hormone (CRH) gene by stress in the hypothalamic paraventricular nucleus**

Coronal brain sections were subjected to *in situ* hybridization using an intronic probe recognizing only heteronuclear RNA (hnRNA, unedited) encoding CRH. (A) Sections derived from stress-free 9-day old pups, sacrificed within 20 s of disturbance, show little CRH hnRNA-specific signal. (B) CRH expression is evident, by 2 min from the onset of stress, and (C) peaks by 15 min. CRH hnRNA levels decline by 30 min from the onset of stress (D), while total CRH-specific mRNA is increased for at least 4 h after stress<sup>29</sup>. Scale bar in (A), 300  $\mu$ m.



**Fig. 3. Distribution and characterization of corticotropin-releasing hormone (CRH)-expressing neurons in the hippocampus**

(A) Immunolabeled CRH-expressing neurons within and adjacent to the pyramidal (s.p.) layer of CA3. (B) A double-labeled neuron, expressing both CRH (brown) and an isoform of glutamic acid decarboxylase (GAD-65), the GABA-synthesizing enzyme (blue-green). (C) An interneuron reacting with antibodies to both CRH (orange) and parvalbumin (plum red). Scale bar, 50  $\mu\text{m}$  in (A), 15  $\mu\text{m}$  in (B) and 20  $\mu\text{m}$  in (C). Abbreviations: s.o., stratum oriens; s.r., stratum radiatum. *Modified, with permission, from Yan et al.*<sup>49</sup>



**Fig. 4. Corticotropin-releasing hormone (CRH) enhances excitability of hippocampal pyramidal neurons**

(A) Reversible increase in the frequency of spontaneous excitatory postsynaptic currents with application of CRH (middle panel). Whole-cell recording of a CA3 pyramidal neuron was performed in voltage-clamp mode ( $-60$  mV). (B) Large, synchronized, variable-latency discharges of CA3 neurons in current-clamp mode (in the presence of bicuculline) are elicited by stimulation of mossy fibers in the presence of CRH. These action-potential discharges are longer-latency, probably polysynaptic, excitatory postsynaptic potentials, and are fully reversed when CRH is removed. Abbreviation: ACSF, artificial cerebrospinal fluid. Modified, with permission, from Hollrigel et al.<sup>67</sup>

**TABLE 1**

Provoked seizures occurring predominantly or exclusively during development

Seizure type	Trigger	Age specificity (human)	Predominant/exclusive	References for animal model
Febrile	Fever	Infants and young children	Exclusive	Baram <i>et al.</i> <sup>3</sup> Holtzman <i>et al.</i> <sup>4</sup>
Hypoxic	Decreased brain oxygenation	Neonates	Predominant	Jensen <i>et al.</i> <sup>5</sup>
Infantile spasms	Multiple ('stressors?')	Infants	Exclusive	None, reviewed by Baram <sup>6</sup>