The Possibility of Neurotoxicity in the Hippocampus in Major Depression: A Primer on Neuron Death

Robert M. Sapolsky

A number of studies indicate that prolonged, major depression is associated with a selective loss of hippocampal volume that persists long after the depression has resolved. This review is prompted by two ideas. The first is that overt neuron loss may be a contributing factor to the decrease in hippocampal volume. As such, the first half of this article reviews current knowledge about how hippocampal neurons die during insults, focusing on issues related to the trafficking of glutamate and calcium, glutamate receptor subtypes, oxygen radical generation, programmed cell death, and neuronal defenses. This is meant to orient the reader toward the biology that is likely to underlie any such instances of neuron loss in major depression. The second idea is that glucocorticoids, the adrenal steroids secreted during stress, may play a contributing role to any such neuron loss. The subtypes of depression associated with the hippocampal atrophy typically involve significant hypersecretion of glucocorticoids, and the steroid has a variety of adverse effects in the hippocampus, including causing overt neuron loss. The second half of this article reviews the steps in this cascade of hippocampal neuron death that are regulated by glucocorticoids. Biol Psychiatry 2000;48:755–765 © 2000 Society of Biological Psychiatry

Key Words: Excitotoxicity, hippocampus, glutamate, calcium, oxygen radicals, glucocorticoids

Introduction

The introduction of the monoamine oxidase inhibitors, now many decades ago, ushered in the era of depression being viewed as a biochemical disorder. There has been unabated interest since then in the neurochemical underpinnings of the disease, as norepinephrine, dopamine, and serotonin have swung in and out of fashion.

More recently, there has been interest in the fact that major and prolonged depression appears to also involve morphological changes in the brain (Bremner et al 2000;

Sheline et al 1996, 1999). In this special issue of *Biological Psychiatry* there will be frequent attention paid to the decrease in depression in the volume of the hippocampus, a structure that plays a vital role in learning and memory, contextual fear conditioning, and neuroendocrine regulation. Such atrophy, of as much as approximately 20% of hippocampal volume, is demonstrable after controlling for total brain volume or volume of the amygdala or temporal lobe. Moreover, atrophy increases with longer durations of depression and persists up to decades after depressions have resolved (Bremner et al 1999; Sheline et al 1996, 1999).

This is a striking finding, prompting obvious interest as to the possible cellular mechanisms underlying it. One possibility to be considered in some articles is that depression induces regression of dendritic processes. Another is that there is inhibition of neurogenesis (a phenomenon whose demonstration in the adult hippocampus, including in humans, is revolutionary [Eriksson et al 1998; Gould et al 1999]). A final possibility is that prolonged depression causes overt loss of hippocampal neurons.

A second idea that will be aired in subsequent articles is that glucocorticoids, the adrenal steroids secreted during stress, contribute to the hippocampal atrophy. The subtypes of depression most associated with hippocampal atrophy involve hypersecretion of glucocorticoids (Sheline et al 1999; Whiteford et al 1987). Moreover, these hormones have various adverse effects that are either most pronounced or are limited to the hippocampus, including 1) inducing regression of dendritic processes; 2) inhibiting neurogenesis; 3) impairing the ability of neurons to survive coincident insults, thereby worsening the neurotoxicity of seizure, hypoxia–ischemia, metabolic poisons, hypoglycemia, and oxygen radical generators; 4) with sufficient exposure to excessive glucocorticoids, neurotoxicity. The last effect is limited to the CA3 cell field in the hippocampus, and in evaluating the mechanisms underlying such neurotoxicity, to be discussed below, it is important to note that this area has no higher concentrations of corticosteroid receptors than some brain regions (reviewed in Gould et al 1999; Reagan and McEwen 1997; Sapolsky 1999). Moreover, selective atrophy of the hippocampus occurs in Cushing syndrome, in which there is hypersecretion of glucocorticoids, and in posttraumatic stress

disorder, in which there are either elevated levels of glucocorticoids (e.g., Pitman and Orr 1990) or enhanced target tissue sensitivity to glucocorticoids (e.g., Yehuda et al 1991; reviewed in Bremner 1999; Sapolsky 1996, in press).

This review is prompted by the possibility that hippocampal atrophy in depression does involve glutamatergic neuron loss, and that glucocorticoid excess plays a role in this loss. Its purpose is twofold: to review current thinking about the mechanisms by which hippocampal neurons die during insults and the facets of such neuron death that are sensitive to glucocorticoids.

Hippocampal Neuron Death: A Broad Outline

The hippocampus is either the most or among the most vulnerable of brain regions to neuron loss arising from seizure, hypoxia–ischemia, and hypoglycemia. This vulnerability has inspired considerable research as to its underlying causes.

From this has emerged something resembling a central dogma (Lee et al 1999) centered around the preponderance of synapses in the hippocampus making use of glutamate as a neurotransmitter. This is the most excitatory neurotransmitter known, and this property, coupled with some nonlinear features of glutamate receptor activation, is key to its normal function. During periods of the repeated stimulation that characterizes learning, glutamate accumulates in the synapse and binds to glutamate receptors. The binding to and ultimate activation of the *N*-methyl-D-aspartate (NMDA) subtype of receptor causes mobilization of free cytosolic calcium. Calcium enters the neuron through NMDA- and voltage-gated channels, as well as being released from intracellular organelles. This calcium mobilization activates the long-term changes in synaptic excitability that probably constitute "memory."

But the neurologic insults just cited all involve an excess of glutamate accumulating in the synapse that, at sufficiently high concentrations, no longer functions as an excitatory neurotransmitter and instead becomes an "excitotoxin." Excess cytosolic calcium is mobilized, producing "promiscuous" overactivity of calcium-dependent enzymes. This produces cytoskeletal degradation, protein malfolding, and oxygen radical generation, which collectively lead to neuron death.

The scenario just described represents the state of knowledge as of about a decade ago, and its broad features still apply. As an important point to emphasize, although hypoxia–ischemia, seizure, and hypoglycemia all activate this pathway, those insults are not mechanistically synonymous. For example, such insults differ as to which glutamate receptors are most central to the neurotoxicity,

what are the main sources of the calcium that enters the cytoplasm, which oxygen radicals are generated, and which cellular compartments are most vulnerable to oxidative damage (for a review, see Lipton 1999). Although the details of these differences are beyond the scope of this review, it should be recalled that the generic pathway of neuron death in response to necrotic insults presented here represents a generalization.

Recent Insights Regarding Glutamatergic Neurotoxicity

I will now describe some of the most interesting and important modifications in this picture that have emerged more recently. Predictably, the bulk of them demonstrate that the "truths" presented above are a bit less certain than originally thought.

Glutamate Accumulation in the Synapse

The levels of a neurotransmitter in the synapse represent a combination of the rate at which it enters that compartment (through neuronal release, most typically) and the rate at which it is removed or degraded. There is increased presynaptic release of glutamate in the hippocampus during the insults, which give rise to excessive synaptic glutamate levels. One important modification in this area in recent years has been the recognition that, if glutamate removal from the synapse cannot keep pace with accumulation, damage will ensue, and that this occurs during insults (Rothstein et al 1996; Volterra et al 1994). It is logical that such uptake may fail to keep pace with glutamate accumulation during an energetic insult, in that glutamate removal from the synapse involves a sodium cotransporter, meaning that an extracellular excess of sodium is required to fuel such uptake. Thus, a crisis that disrupts energy production (as in hypoxia–ischemia or hypoglycemia) or depletes energy stores because of high energy demands (such as a seizure) will certainly limit the ability of the sodium/potassium/adenosine triphosphatase to adequately maintain extracellular sodium concentrations.

As a second modification of this picture, glutamate is removed from the synapse through reuptake by the presynaptic neuron, as well as by uptake by glia. Such glia then convert glutamate to glutamine, transferring it back to the presynaptic neuron for reconversion to glutamate. This glial route accounts for the majority of uptake (Hertz et al 1993). Thus, insofar as failure of uptake mechanisms contributes to extracellular glutamate accumulation, this is predominately a glial phenomenon.

An additional facet of this story has recently been

appreciated. As just discussed, glutamate uptake from the synapse is driven by high extracellular sodium concentrations, and a collapse of that gradient impairs uptake. With sufficient energetic collapse in the face of severe insults, neuronal membranes will depolarize to such an extent as to produce a preponderance of *intra*cellular sodium. Not only will glutamate removal from the synapse fail, there will be reversal of the pump. Thus, to the extent that insults involve increased secretion of glutamate, only a small percentage of that is due to exocytotic release of vesicular glutamate. The bulk instead is due to pump reversal and the efflux of the large cytosolic pools of glutamate (Rossi et al 2000; in glia as well: Longuemare and Swanson 1995).

Finally, while glutamate is an obvious prerequisite for glutamatergic neurotoxicity, such death does not necessarily require glutamate *excess.* "Weak excitotoxic" death is the circumstance where during a period where energetics is sufficiently compromised even normal glutamate concentrations can be too much of an excitotoxic load for a neuron (cf. Turski and Turski 1993). This shifts the emphasis to the next step in this cascade (viz., postsynaptic sensitivity to glutamate).

Glutamate Receptors

There are a variety of ionotropic and metabotropic glutamate receptors. The former include the calcium-conducting NMDA receptor as well as non-NMDA receptors, which, traditionally, were thought to solely conduct sodium. As noted, it was originally believed that activation of the NMDA receptor during glutamate excess was necessary for excitotoxicity. This gave rise to an optimism that pharmacologic inhibition of NMDA receptors would protect dramatically against insults. Although blockade of such receptors can be quite protective against milder insults, such as focal ischemic damage or prolonged exposure to moderate inhibition of neuronal energetics (Fiskum et al 1999), there have been some disappointments in this regard (Lee et al 1999).

One reason for this is that the conductance of NMDA receptors is often quite inhibited at times when glutamate levels are their highest. It is now known that the NMDA receptor is constrained by numerous negative feedback loops. For example, the increasing cytosolic calcium concentrations following NMDA receptor activation lead to an inhibitory feedback loop upon that receptor (Lieberman and Mody 1994). In addition, protons, generated by the anaerobic metabolism that occurs during hypoxia–ischemia, inhibit NMDA receptor activation (Traynelis and Cull-Candy 1990), such that mild acidosis is neuroprotective (a very counterintuitive finding in the field of neurology; Tombaugh and Sapolsky 1993). As a third

example, calcium excess during insults leads to generation of nitric oxide, which can nitrosylate the NMDA receptor, thereby decreasing its activity (Lipton et al 1993). Finally, dephosphorylation of the receptor during hypoxia–ischemia will also decrease activity (Lobner and Lipton 1993).

Thus, glutamatergic insults set in motion events that act protectively to decrease NMDA receptor sensitivity; as such, the NMDA receptor is often far from its most responsive precisely during times when synaptic glutamate concentrations are their highest. This probably explains why NMDA receptor antagonists have proven to be less protective against some insults than anticipated. Nonetheless, there must still be factors that bias toward NMDA receptor–mediated neuron death. This is because of the phenomenon of weak excitotoxicity, when heavy glutamate exposure increases NMDA receptor sensitivity such that subsequent exposure to even normal levels of glutamate can prove neurotoxic (for a review, see Tombaugh and Sapolsky 1993).

Along with the decreased emphasis on the NMDA receptor as the key to glutamatergic neurotoxicity has come the recognition that other glutamate receptors play more damaging roles than originally thought. Attention has focused on the ionotropic AMPA receptor. This receptor consists of an array of differing subunits and, in contrast to the dogma about only the NMDA receptor conducting calcium, AMPA-gated channels also conduct calcium when they lack the GluR2 subunit. Critically, various hippocampal insults decrease GluR2 expression (Gorter et al 1997), potentially explaining instances in which AMPA receptor antagonists are more neuroprotective than are NMDA receptor antagonists (Pellegrini-Giampietro et al 1997; Yatsugi et al 1996).

Calcium Mobilization

In the mid 1980s, an influential model posited that sodium and chloride influx was the proximal cause of glutamatergic neuron death. This ionic movement drove sufficient water into the neuron to cause swelling and bursting (Rothman 1985). This was ultimately shown to be somewhat of an artifact of the phenomenon being studied in vitro. In the two-dimensional environment of the cell culture monolayer, cell somas are less constrained and can actually burst. In contrast, the picture in the three-dimensionally constrained in vivo world is instead one of somas collapsing into pyknosis. This shift away from the chloride model came around the same time as the recognition of the dependency of glutamatergic neurotoxicity on calcium mobilization into the cytoplasm (Choi 1988). Central to this shift in emphasis were the neuroprotective effects in experimental models of necrotic insults of removing extracellular calcium, administering calcium chelators, or

overexpressing calcium-binding proteins (Choi 1995; Meier et al 1997, 1998). These findings generated considerable optimism that calcium channel blockers or calcium chelators would prove to be clinically neuroprotective as well.

This has proven disappointing. In addition, calcium imaging studies have also generated a disappointing inability to demonstrate what seemed an obvious corollary of the "calcium hypothesis." This was the idea that, on a rather straightforward level, the greater the amount of free cytosolic calcium mobilized in a neuron by an insult, the greater the likelihood of death. This is usually not the case (for a review, see Lipton and Rosenberg 1994).

The solution to this puzzle is logical to a cell biologist. The cytoplasm of a cell, especially a neuron, is not an undifferentiated water balloon filled with enzymes and organelles floating here or there. Instead, it is highly structured and compartmentalized, where local concentrations of cytosolic calcium, typically in dendritic spines, predict likelihood of neuron death (Bindokas and Miller 1995). Moreover, it is not only the amount of calcium in cytosolic microenvironments that is critical, but the source of such calcium as well—calcium derived from the extracellular environment rather than organelles, calcium derived from mitochondria rather than the endoplasmic reticuluum, and so on (see, e.g., Sattler et al 1998, concerning the particularly damaging role of calcium entering via the NMDA receptor–gated channel). As one example of how this might work, calcium uptake by mitochondria is an energetically costly high-capacity system that is activated during severe insults, and calcium released by mitochondria is more likely to trigger a futile and energetically costly cycle of release and uptake.

Postcalcium Degenerative Events

As noted, the cytosolic calcium excess causes promiscuous overactivation of varied damaging calcium-dependent events. Around a decade ago, it was cytoskeletal degradation that received the most attention. This reflected the historical roots of studying neuron death with microscopic techniques, which revealed little more than that dead neurons had a pyknotic morphology. This implied a dramatic destruction of the cytoskeletal proteins responsible for the cytoarchitecture of a healthy neuron. Thus, there was interest in the activation of calcium-dependent calpain following insults, and the ability of this enzyme to cleave the cytoskeletal protein spectrin (Lee et al 1991). Subsequent work has shown that calpain inhibitors can protect against ischemic damage (Bartus et al 1994).

With the passing years, there has been increasing interest in the additional role played by the calcium-dependent generation of oxygen radicals (Coyle and Put-

tfarcken 1993). This is probably due to three threads of research:

1. Biochemical insight as to how calcium excess generates oxygen radicals. This includes induction of nitric oxide synthase and the generation of nitric oxide, the generation of xanthine oxidase, the activation of phospholipases that liberate arachidonic acid from membranes, and the disruption of mitochondrial function.
2. The development of techniques with sufficient sensitivity to detect the small and brief bursts of oxygen radical accumulation following insults.
3. The demonstration that such quantities of oxygen radicals are deleterious.

With this view that oxidative routes of damage are important contributors to damage following insults have come questions as to which oxygen radicals are most damaging, and whether it is oxidative damage to lipids, proteins, or nucleic acids that is most consequential.

One additional development in this field has been the subject of intense research. Hippocampal neuron death after various insults has traditionally been termed *necrotic*, a mode of death involving chaotic and idiosyncratic damage. This term would be contrasted to a subtype of programmed cell death (viz., apoptosis). Such death is linear, where a stereotyped sequence of suicide steps is activated. Such apoptosis was thought to occur in the nervous system only during development.

More sensitive means to detect apoptosis, developed in the early 1990s, produced the surprising observation that glutamatergic insults trigger apoptosis in a subset of moribund neurons (Green and Reed 1998; Roy and Sapolsky 1999). The transition point between necrotic and apoptotic neuron death remains uncertain, but seems to occur somewhere around the calcium excess and oxygen radical generation. As one contributing factor, this is the stage at which proapoptotic proteins such as BAX form pores in mitochondrial membranes. In addition, mitochondria are a high-capacity sink for sequestering calcium and, as the mitochondria fill with calcium, water follows, causing osmotic swelling (termed *microvacuolization* by neuropathologists). This brings parts of the inner mitochondrial membrane into contact with the outer membrane, causing pore formation. Some combination of the effects of BAX and of swelling releases cytochrome c and other proapoptotic factors from the mitochondria, triggering the cascade of programmed cell death (Adams and Cory 1998; Green and Reed 1998).

Although the occurrence of apoptosis after necrotic insults was unexpected, it is actually logical. Apoptosis involves alterations of surface antigens, regression of neuronal processes, rounding up of the cell body, and

DNA cleavage, all of which facilitate the clean engulfing of the neuron by reactive microglia, minimizing inflammation. In contrast, in necrotic neuron death the cell membrane ruptures, releasing proinflammatory contents of the neuron. Critically, apoptosis is an active, energy-consuming process, explaining why only a subset of neurons can carry it out (Roy and Sapolsky, 1999); younger neurons and those with more energy are the more likely to do so.

Thus, the dominant features of calcium-dependent degeneration involve cytoskeletal damage and oxidative damage and, in a subset of neurons, the triggering of apoptosis.

Energy as a Key Modulator of Glutamatergic Neuron Death

As just discussed, energy availability plays a role in determining whether neurons die with a necrotic or apoptotic phenotype. Energy availability modulates glutamatergic death in general (Beal et al 1993; Turski and Turski 1993); for example, a paucity of energy causes glutamate concentrations that would normally be excitatory to become excitotoxic (a central idea of "weak" excitotoxicity). The specific steps of glutamatergic injury that are most energy sensitive are coming to be appreciated. One key one, as discussed, is the ability of neurons and glia to remove glutamate from the synapse, which depends upon the energetically costly process of maintaining transmembrane ionic gradients. There are also considerable energetic costs in removing calcium from the cytoplasm of postsynaptic neurons. Calcium extrusion from the cell or uptake into organelles relies upon pumps that are either directly driven by adenosine triphosphate (ATP) hydrolysis, or by ionic gradients. Thus, energy availability modulates the efficacy with which a neuron can contain calcium excesses.

Neuronal Defenses against Glutamatergic Insults

Neurons are not passively buffered by insults, but instead mobilize varied defenses. A few are familiar—the active removal of glutamate from the synapse, or calcium from the cytoplasm, can be viewed as a "defense." This is so apparent, however, that it is difficult to perceive these actions in that context. A number, however, are less obvious. These include:

- Adaptations that supply more energy. For example, there is upregulation of the glucose transporter in the nervous system following some insults (cf. Vannucci et al 1995). In addition, the uptake of cytosolic calcium by mitochondria during insults serves as a signal to enhance oxidative phosphorylation (McCor-

mack and Denton 1993). Moreover, during insults neurons make use of lactate for metabolism, and glia increase lactate synthesis and secretion for neuronal utilization (Pellerin and Magistretti 1994).

- The synthesis of heat shock proteins, which have a variety of protective effects in endangered neurons (cf. Vass et al 1988).

- The generation and release of adenosine (Manzoni et al 1994; Young and Dragunow 1994). During an energetic insult, ATP is consumed, producing, successively, adenosine diphosphate, adenosine monophosphate, and, ultimately, adenosine. By definition, adenosine accumulation signals energetic crisis. Adenosine is then released, travels as a retrograde neurotransmitter, and inhibits glutamate release, constituting a transynaptic negative feedback loop.

- As discussed, there are intracellular negative feedback loops upon the NMDA receptor, involving protons, nitric oxide, and calcium.

- Neurons can be protectively hyperpolarized by a number of potassium channels whose openings are triggered by signals of neuronal crisis. Thus, there are rectifying potassium channels triggered by an excess of cytosolic calcium or by ATP depletion (Heurteaux et al 1995).

- Finally, there is protective upregulation of antioxidant enzymes following some insults (McIntosh et al 1998).

This partial list gives a flavor of the range of defenses mobilized. Although there is increasing understanding of these individual adaptations, there does not yet exist a coherent picture of neuronal defenses in general. This emerging subject requires a greater understanding of the differing thresholds of injury at which various defenses are first mobilized, as well as of when the ceiling of their protective potentials is reached. The latter would constitute important information in deciding which particular defense might be a good candidate to try to enhance.

This brief review was meant to summarize some recent and important findings concerning how a hippocampal neuron dies following a necrotic insult. As noted, this is a prelude to discussions in some later articles concerning the possibility that hippocampal atrophy in major depression is a consequence of neuron loss, a process mediated in part by glucocorticoids. In the remainder of this review I consider some of the steps discussed above that are modulated by glucocorticoids.

Glucocorticoids and Their Adverse Effects in the Hippocampus

The hippocampus is one of the principle glucocorticoid target sites in the brain, with ample concentrations of

corticosteroid receptors. When glucocorticoid levels rise into the mild stress range for a few hours, they can enhance cognition by facilitating aspects of synaptic plasticity in the hippocampus (for a review, see McEwen and Sapolsky 1995). This has been speculated to make some teleologic sense—memory is not automatically consolidated concerning all events occurring in the environment of an organism. Instead, it is adaptive for there to exist a mechanism by which an organism pays attention to emotionally significant events, and those glucocorticoid effects contribute to this phenomenon; however, in the face of major and prolonged glucocorticoid secretion, that same hippocampal sensitivity to the steroid ultimately has deleterious effects including, eventually, overt neuron loss.

The first evidence that glucocorticoids could be directly neurotoxic in the hippocampus came more than 30 years ago. More recently, there has been a steady rate of research examining the phenomenon, along with the related ability of glucocorticoids to augment the neurotoxicity of other hippocampal insults. These studies have identified steps in the glutamatergic cascade just reviewed that are directly activated by glucocorticoids or, when activated by a necrotic insult, are exacerbated by glucocorticoids. These studies have also identified glucocorticoid actions that indirectly modulate the glutamatergic cascade.

Effects upon Neuronal Energetics

One such indirect glucocorticoid effect concerns neuronal energetics. Glucocorticoids have long been known to inhibit glucose transport in various peripheral tissues; this can be viewed as a strategy to divert energy toward exercising muscle during a stressor (Munck 1971). The inhibition involves both translocation of glucose transporters off of the cell membrane and a decrease in the synthesis of new transporters.

A similar inhibition has been reported in the hippocampus, with glucocorticoids decreasing glucose uptake by cultured neurons and glia and decreasing local cerebral glucose utilization in the hippocampus in vivo (Bryan and King 1988; Doyle et al 1993; Freo et al 1992; Horner et al 1990; Kadekaro et al 1988; Virgin et al 1991; but see Seckl et al 1991 for contradiction). The effect is small—glucocorticoids inhibit on the order of 70–80% of glucose uptake in an adipocyte but only 20–25% in the hippocampus. Nonetheless, it has been speculated that this is sufficient to compromise the ability of a neuron to carry out the costly task of defending itself against an insult. Supporting this, glucocorticoids accelerate the decline in hippocampal ATP concentrations, metabolism, and mitochondrial potentials during insults (for a review, see Sapolsky 1999). Furthermore, as will be reviewed shortly, some glucocorticoid effects upon the trafficking of gluta-

mate and calcium are secondary to the energetic effects of the steroid.

A related literature suggests that such glucocorticoid effects upon glucose transport can significantly modulate neuronal survival. Viral vectors have been used to overexpress the Glut-1 glucose transporter in neurons as a gene therapy strategy. Such overexpression enhances glucose uptake, along with preserving ATP concentrations and metabolism during insults. In addition, neurons are now more capable of removing glutamate from the synapse and of extruding calcium from the cytoplasm during insults. Finally, glucose transporter overexpression decreases the neurotoxicity of various hippocampal insults (for a review, see Sapolsky and Steinberg 1999). Thus, gene transfer techniques that increase glucose transport in the hippocampus buffer against neurotoxicity, suggesting that the ability of glucocorticoids to decrease glucose transport plays an important role in the ability of the steroid to worsen the neurotoxicity of insults.

Effects upon Glutamate Accumulation

Both stress and glucocorticoids increase glutamate concentrations in the hippocampal synapse (Moghaddam 1993; Moghaddam et al 1994; Lowy et al 1994). Furthermore, glucocorticoids selectively increase glutamate accumulation in response to excitotoxic insults both in hippocampal cultures and in the hippocampus in vivo (Chou et al 1994; Stein-Behrens et al 1992, 1994a). Raising steroid levels from the low basal range to those typically triggered by excitotoxic insults increases glutamate levels fourfold (Stein-Behrens et al 1994a). In the in vitro demonstration of this, the hormone effect was due more to decreasing glutamate removal from the synapse than to increasing release (Chou et al 1994). These glucocorticoid effects appear to arise, at least in part, from the disruptive energetic effects of the steroid. As evidence, energy supplementation blunts the ability of glucocorticoids to augment glutamate levels (Stein-Behrens et al 1992); this fits well with the marked energy dependency of glutamate uptake mechanisms. Finally, glucocorticoids prolong glutamate accumulation postinsult (Lowy et al 1995).

Effects upon Calcium Concentrations

Glucocorticoids increase the free cytosolic calcium load in the hippocampus as well. Insofar as the hormone raises glutamate levels during insults, that should also enhance calcium mobilization. There appear to be direct effects upon calcium trafficking as well. The hormone increases free cytosolic calcium concentrations basally in cultured hippocampal neurons (i.e., under circumstances in which basal glutamate concentrations in the synapse are probably not being elevated; Elliott and Sapolsky 1992, 1993).

Moreover, it increases voltage-dependent calcium conductance and calcium-dependent afterhyperpolarizations and prolongs calcium spike duration in the CA1 cell field of the hippocampus (e.g., Joels and de Kloet 1989; Kerr et al 1992; Landfield and Pitler 1984). Although glucocorticoids both increase the calcium mobilized into the cytosolic compartment and decrease the removal, it is the latter component that appears more sensitive (Elliott and Sapolsky 1992, 1993). As a mechanism to explain the latter, glucocorticoids inhibit transcription of the calcium–ATPase pump, which plays a key role in extruding cytosolic calcium (Bhargava et al, in press). Finally, glucocorticoids also increase the extent to which calcium is mobilized in response to insults (Elliott and Sapolsky 1992, 1993; Goodman et al 1996).

Thus, glucocorticoids increase the basal cytosolic calcium load on a hippocampal neuron and worsen the response to an insult, both through direct postsynaptic effects and through indirectly increasing the glutamatergic tone impinging on the neuron. Some of the steroid actions on calcium levels are probably secondary to the energetic effects already discussed, in that they can be blunted with energy supplementation (Elliott and Sapolsky 1993; Elliott et al 1993); however, some effects are seemingly energy independent (such as some electrophysiologic effects [de Kloet et al 1999], the inhibition of transcription of the calcium–ATPase [Bhargava et al, in press], and inhibition of a signal transduction pathway that inhibits calcium currents [Kolasa et al 1992]). Finally, as a measure of the deleterious consequences of glucocorticoids elevating free cytosolic calcium concentrations, gene therapy strategies that do the opposite (by overexpressing calcium binding proteins) buffer hippocampal neurons from neurotoxicity (Kindy et al 1996; Meier et al 1997, 1998; Phillips et al 1999).

Effects on Calcium-Dependent Degenerative Events

Given glucocorticoid effects on calcium dynamics, it is not surprising that both the hormone and stress worsen calcium-dependent degenerative events such as cytoskeletal proteolysis and tau immunoreactivity (Elliott et al 1993; Stein-Behrens et al 1994b). These effects are at least partially blunted by energy supplementation.

The hormone also worsens oxygen radical accumulation during insults, an effect likely to arise, at least in part, from the ability of glucocorticoids to decrease the activity of some antioxidants (McIntosh et al 1998). This effect may have an energetic component as well; nicotinamide adenine dinucleotide phosphate (NADPH) is a critical cofactor in generating reduced glutathione, the electron acceptor for the antioxidant glutathione peroxidase, and glucocorticoids may disrupt those various steps by limiting NADPH availability during insults (McIntosh et al, in preparation). As a measure of the potential importance of this particular glucocorticoid effect, overexpression of antioxidant enzymes can be neuroprotective (Kindy et al 1996).

Effects on Neuronal Defenses

Insofar as glucocorticoids impair glutamate removal from the synapse and calcium extrusion from the postsynaptic cytoplasm, and blunt the compensatory increase in antioxidant enzyme activity during insults, the hormone can be categorized as disrupting some neuronal defenses. As an additional example of this, glucocorticoids blunt the synthesis and efficacy of the heat shock protein hsp72 during insults (Ajilore et al, submitted), and as a measure of the potentially endangering consequences of this effect, hsp72 overexpression is neuroprotective against an array of insults (Yenari et al 1998).

Glucocorticoid Neurotoxicity versus Endangerment

As discussed, among the adverse effects of glucocorticoids in the hippocampus, they can 1) be directly toxic to neurons, with sufficient hormone excess, or 2) not be directly toxic but, nonetheless, increase the neurotoxicity of various hippocampal insults. The preceding sections regarding glucocorticoid action have been heavily built around interactions between the hormone and the various steps activated in neurons in response to necrotic insults. As such, they have provided a fair amount of insight into how glucocorticoids can worsen the toxicity of hypoxia–ischemia, seizure, or hypoglycemia; however, there is no evidence that such insults occur in the context of depression. Of the adverse glucocorticoid actions just reviewed, how many occur in the absence of simultaneous necrotic insults, and thus may even be plausible candidates for explaining the volume loss in depression?

The inhibition of glucose transport by glucocorticoids occurs without a coincident insult, and thus initially appears to be a candidate mechanism; however, it should be recalled that this effect is quite small, and thus, although the hormone worsens the decline in ATP levels in hippocampal neurons and glia during insults, it does not lower basal, unchallenged levels of ATP (Lawrence and Sapolsky 1994; Tombaugh and Sapolsky 1992).

The glucocorticoid effects on glutamate accumulation probably also do not occur outside the context of a coincident insult. As evidence, although the hormone worsens extracellular glutamate accumulation in vivo shortly after insertion of a microdialysis probe, it does not when the probe has been inserted a few days previously (Lowy et al 1995), suggesting that the former represents an

interaction between the hormone and the insult of probe insertion into the brain.

The effects of the steroid upon calcium concentrations, in contrast, do appear to occur basally. Glucocorticoids elevate free cytosolic calcium concentrations in vitro in the absence of an insult, and the effects upon calcium conductance and upon activity of the calcium–ATPase pump are demonstrable without an insult.

Finally, the bulk of glucocorticoid effects on calcium-dependent degenerative events and upon neuronal defenses have only been demonstrated in the context of a coincident insult.

Conclusions

This review has had two goals. The first has been to orient the reader toward current thinking about how hippocampal neurons die in response to insults. As was seen, a number of themes dominate these pathways of neurodegeneration. These include the idea that normative cellular physiology that mediates a critical aspect of cognition (learning and memory) can, when writ large, become pathophysiology. Another is that with a neurotransmitter as excitatory as glutamate, and with an ion as multifaceted as calcium, their regulation within the synapse and cytoplasm, respectively, is extremely expensive and thus vulnerable. As a corollary of this, the high metabolic demands of brain tissue, particularly in the hippocampus, mean that instances of energetic limits will have broadly deleterious effects. And as a corollary of that, the heavily aerobic features of neuronal metabolism mean that the chaos of injury will carry the special risk of oxygen radical accumulation.

These features of hippocampal neuronal vulnerability may help explain why it is that in prolonged major depression, as well as in Cushing syndrome and posttraumatic stress disorder following chronic traumas, there is volume loss that is either unique to the hippocampus or uniquely persistent. A number of subsequent articles will consider the possibility of overt neuron loss underlying the hippocampal volume loss in depression. It should be emphasized, however, that there is no current evidence that there is hippocampal neuron loss during depression, with or without pathologic activation of glutamatergic pathways; however, the persistence of volume loss for decades after the resolution of depressions (Sheline et al 1996, 1999) must raise the possibility of neuron loss, and given the neurochemical nature of the hippocampus, any instance of neuron loss in the hippocampus must raise the possibility of it being glutamatergic. Obviously, a great deal of work is needed in this area.

This review has also raised the issue of whether glucocorticoids play some role in such putative neuron loss, a possibility that will also be raised in subsequent articles. The possibility is made more pressing by the fact that it is the subtypes of depression most associated with atrophy that tend toward the highest rates of hypercortisolism (Sheline et al 1999). The hormone worsens a variety of steps relevant to understanding how it can exacerbate the massive amounts of neuron death occurring within days of a necrotic neurologic insult; however, glucocorticoid effects on this pathway in the absence of an insult are far more subtle, seemingly involving an elevation of basal free cytosolic calcium concentrations. One can readily construct scenarios in which this glucocorticoid action, if occurring over the course of years because of prolonged hypercortisolemic depression, could increase the vulnerability of neurons to extremely mild challenges, such as the transient hypoglycemia of a skipped meal or the transient hypoxia–ischemia of brief vasospasm. One can also readily appreciate the difficulty of such future studies.

References

Adams J, Cory S (1998): The bcl-2 protein family: Arbiters of cell survival. *Science* 281:1322–1326.

Ajilore O, Fink S, Sapolsky R (submitted): Effects of glucocorticoids on heat-shock protein induction and protection in rat hippocampus.

Bartus R, Baker K, Heiser A, Sawyer S, Dean R, Elliott P, Straub J (1994): Postischemic administration of AK275, a calpain inhibitor, provides substantial protection against focal ischemic brain damage. *J Cereb Blood Flow Metab* 14:537–544.

Beal M, Hyman B, Koroshetz W (1993): Do defects in mitochondrial energy metabolism underlie the pathology of neurodegenerative diseases? *Trends Neurosci* 16:125–131.

Bhargava A, Meijer O, Dallman M, Pearce D (in press): Plasma membrane calcium pump isoform 1 gene expression is repressed by corticosterone and stress in rat hippocampus. *J Neurosci.*

Bindokas V, Miller R (1995): Excitotoxic degeneration is initiated at non-random sites in cultured rat cerebellar neurons. *J Neurosci* 15:6999–7011.

Bremner J (1999): Does stress damage the brain? *Biol Psychiatry* 45:797–805.

Bremner J, Narayan M, Anderson E, Staib L, Miller H, Charney D (2000): Hippocampal volume reduction in major depression. *Am J Psychiatry* 157:115–118.

Bryan R, King J (1988): Glucocorticoids modulate the effect of plasma epinephrine on regional glucose utilization. *Soc Neurosci Abstr* 399:11.

Choi D (1988): Calcium-mediated neurotoxicity: Relationship to specific channel types and role in ischemic damage. *Trends Neurosci* 11:465–469.

Choi D (1995): Calcium: Still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci* 18:58–60.

Chou Y, Lin W, Sapolsky R (1994): Glucocorticoids increase extracellular [3H]D-aspartate overflow in hippocampal cultures during cyanide-induced ischemia. *Brain Res* 654:8–14.

Coyle J, Puttfarcken P (1993): Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262:689–695.

de Kloet E, Oitzl M, Joels M (1999): Stress and cognition: Are corticosteroids good or bad guys? *Trends Neurosci* 22:422–426.

Doyle P, Rohner-Jeanrenaud F, Jeanrenaud B (1993): Local cerebral glucose utilization in brains of lean and genetically obese (fa/fa) rats. *Am J Physiol* 264:E29–E36.

Elliott E, Mattson M, Vanderklish P, Lynch G, Chang I, Sapolsky R (1993): Corticosterone exacerbates kainate-induced alterations in hippocampal tau immunoreactivity and spectrin proteolysis in vivo. *J Neurochem* 61:57–67.

Elliott E, Sapolsky R (1992): Corticosterone enhances kainic acid-induced calcium mobilization in cultured hippocampal neurons. *J Neurochem* 59:1033–1040.

Elliott E, Sapolsky R (1993): Corticosterone impairs hippocampal neuronal calcium regulation: Possible mediating mechanisms. *Brain Res* 602:84–90.

Eriksson P, Perfilieva E, Bjork-Eriksson T, Alborn A, Nordborg C, Peterson D, Gage F (1998): Neurogenesis in the adult human hippocampus. *Nat Med* 4:1313–1317.

Fiskum G, Murphy A, Beal M (1999): Mitochondria in neurodegeneration: Acute ischemia and chronic neurodegenerative diseases. *J Cereb Blood Flow Metab* 19:351–369.

Freo U, Holloway H, Kalogeras K, Rapoport S, Soncrant T (1992): Adrenalectomy or metyrapone-pretreatment abolishes cerebral metabolic responses to the serotonin agonist DOI in the hippocampus. *Brain Res* 586:256–264.

Goodman Y, Bruce A, Cheng B, Mattson M (1996): Estrogens attenuate excitotoxicity, oxidative injury, and amyloid B-peptide toxicity in hippocampal neurons. *J Neurochem* 66:1836–1844.

Gorter J, Petrozzino J, Aronica E, Rosenbaum D, Opitz T, Bennett M, et al (1997): Global ischemia induces downregulation of Glur2 mRNA and increases AMPA receptor-mediated Ca2+ influx in hippocampal CA1 neurons of gerbil. *J Neurosci* 17:6179–6188.

Gould E, Reeves A, Graziano M, Gross C (1999): Neurogenesis in the neocortex of adult primates. *Science* 286:548–552.

Green D, Reed J (1998): Mitochondria and apoptosis. *Science* 281:1309–1312.

Hertz L, Kvamme E, McGreer E, Schousboe E, editors (1993): *Glutamine, Glutamate and GABA in the Central Nervous System.* New York: Liss.

Heurteaux C, Lauritzen I, Widmann C, Lazdunski M (1995): Essential role of adenosine, adenosine Al receptors and KATP channels in cerebral ischemic preconditioning. *Proc Natl Acad Sci U S A* 92:4666–4670.

Horner H, Packan D, Sapolsky R (1990): Glucocorticoids inhibit glucose transport in cultured hippocampal neurons and glia. *Neuroendocrinology* 52:57–64.

Joels M, de Kloet E (1989a): Effects of glucocorticoids and norepinephrine on the excitability in the hippocampus. *Science* 245:1502–1505.

Joels M, de Kloet E (1989b): Mineralocorticoid receptor-mediated changes in membrane properties of rat CA1 pyramidal neurons in vitro. *Proc Natl Acad Sci U S A* 87:4495–4498.

Kadekaro M, Masanori I, Gross P (1988): Local cerebral glucose utilization is increased in acutely adrenalectomized rats. *Neuroendocrinology* 47:329–334.

Kerr D, Campbell L, Thibault O, Landfield P (1992): Hippocampal glucocorticoid receptor activation enhances voltage-dependent calcium conductances: Relevance to brain aging. *Proc Natl Acad Sci U S A* 89:8527–8531.

Kindy M, Yu J, Miller R, Roos R, Ghadge G (1996): Adenoviral vectors in ischemic injury. In: Krieglstein J, editor. *Pharmacology of Cerebral Ischemia.*

Kolasa K, Song L, Jope R (1992): Adrenalectomy increases phosphoinositide hydrolysis induced by norepinephrine or excitatory amino acids in rat hippocampal slices. *Brain Res* 579:128–134.

Landfield P, Pitler T (1984): Prolonged calcium-dependent afterhyperpolarizations in hippocampal neurons of aged rats. *Science* 226:1089–1092.

Lawrence MS, Sapolsky R (1994): Glucocorticoids accelerate ATP loss following metabolic insults in cultured hippocampal neurons. *Brain Res* 646:303–306.

Lee J, Zipfel G, Choi D (1999): The changing landscape of ischaemic brain injury mechanisms. *Nat Suppl* 399:A7–A14.

Lee K, Frank S, Vanderklish P, Arai A, Lynch G (1991): Inhibition of proteolysis protects hippocampal neurons from ischemia. *Proc Natl Acad Sci U S A* 88:7233–7237.

Lieberman D, Mody I (1994): Regulation of NMDA channel function by endogenous calcium-dependent phosphatase. *Nature* 369:235–239.

Lipton P (1999): Ischemic cell death in brain neurons. *Physiol Rev* 79:1431–1568.

Lipton S, Choi Y, Pan Z, Lei S, Chen H, Sucher N, et al (1993): A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364:626–632.

Lipton S, Rosenberg P (1994): Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med* 330:613–622.

Lobner D, Lipton P (1993): Intracellular calcium levels and calcium fluxes in the CA1 region of the rat hippocampal slice during in vitro ischemia: Relationship to electrophysiological cell damage. *J Neurosci* 13:4861–4871.

Longuemare M, Swanson R (1995): Amino acid release from astrocytes during energy failure by reversal of sodium-dependent uptake. *J Neurosci Res* 40:379–386.

Lowy M, Wittenberg L, Novotney S (1994): Adrenalectomy attenuates kainic acid-induced spectrin proteolysis and heat shock protein 70 induction in hippocampus and cortex. *J Neurochem* 63:886–894.

Lowy M, Wittenberg L, Yamamoto B (1995): Effect of acute

stress on hippocampal glutamate levels and spectrin proteolysis in young and aged rats. *J Neurochem* 65:268–274.

Manzoni O, Manabe T, Nicoll R (1994): Release of adenosine by activation of NMDA receptors in the hippocampus. *Science* 265:2098–2101.

McCormack J, Denton R (1993): Mitochondrial calcium transport and the role of intramitochondrial calcium in the regulation of energy metabolism. *Dev Neurosci* 15:165–173.

McEwen B, Sapolsky R (1995): Stress and cognitive function. *Curr Opin Neurobiol* 5:205–216.

McIntosh LJ, Cortopassi K, Sapolsky R (1998): Glucocorticoids may alter antioxidant enzyme capacity in the brain: Kainic acid studies. *Brain Res* 791:215–222.

Meier T, Ho D, Park T, Sapolsky R (1998): Gene transfer of calbindin D28K cDNA via herpes simplex virus amplicon vector decreases calcium ion mobilization and enhances neuronal survival following glutamatergic challenge but not following cyanide. *J Neurochem* 79:1013–1023.

Meier T, Ho D, Sapolsky R (1997): Increased expression of calbindin D28K via herpes simplex virus amplicon vector decreases calcium ion mobilization and enhances neuronal survival following hypoglycemic challenge. *Neurochemistry* 69:1039–1047.

Moghaddam B (1993): Stress preferentially increases extraneuronal levels of excitatory amino acids in the prefrontal cortex: Comparison to hippocampus and basal ganglia. *J Neurochem* 60:1650–1657.

Moghaddam B, Bolinao M, Stein-Behrens B, Sapolsky R (1994): Glucocorticoids mediate the stress-induced accumulation of extracellular glutamate. *Brain Res* 655:251–254.

Munck A (1971): Glucocorticoid inhibition of glucose uptake by peripheral tissues: Old and new evidence, molecular mechanisms, and physiological significance. *Perspect Biol Med* 14:265–269.

Pellegrini-Giampietro D, Gorter J, Bennett M, Zukin R (1997): The GluR2 hypothesis: Calcium-permeable AMPA receptors in neurological disorders. *Trends Neurosci* 20:464–470.

Pellerin L, Magistretti P (1994): Glutamate uptake into astrocytes stimulate aerobic glycolysis: A mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A* 91:10625–10629.

Phillips R, Meier T, Giuli L, McLaughlin J, Ho D, Sapolsky R (1999): Calbindin D28K gene-transfer via herpes simplex virus amplicon vector decreases hippocampal damage in vivo following neurotoxic insults. *J Neurochem* 73:1200–1205.

Pitman R, Orr S (1990): Twenty-four hour urinary cortisol and catecholamine excretion in combat-related PTSD. *Biol Psychiatry* 27:245–247.

Reagan L, McEwen B (1997): Controversies surrounding glucocorticoid-mediated cell death in the hippocampus. *J Chem Neuroanat* 13:149–167.

Rossi D, Oshima T, Attwell D (2000): Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature* 403:316–321.

Rothman S (1985): The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *J Neurosci* 5:1483–1489.

Rothstein J, Dykes-Hoberg M, Pardo C, Bristol L, Jin L, Kuncl R, et al (1996): Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16:675–686.

Roy M, Sapolsky R (1999): Neuronal apoptosis in acute necrotic insults: Why is this subject such a mess? *Trends Neurosci* 22:419–422.

Sapolsky R (1996): Why stress is bad for your brain. *Science* 273:749–750.

Sapolsky R (1999): Stress, glucocorticoids and their adverse neurological effects: Relevance to aging. *Exp Gerontol* 34:721–732.

Sapolsky R (in press): Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch Gen Psychiatry.*

Sapolsky R, Steinberg G (1999): Gene therapy for acute neurological insults. *Neurology* 10:1922–1931.

Sattler R, Charlton MP, Hafner M, Tymianski M (1998): Distinct influx pathways, not calcium load, determine neuronal vulnerability to calcium neurotoxicity. *J Neurochem* 71:2349–2364.

Seckl J, Kelly P, Sharkey J (1991): Glycyrrhetinic acid, an inhibitor of 11beta-hydroxysteroid dehydrogenase, alters local cerebral glucose utilization in vivo. *J Steriod Biochem Mol Biol* 39:777–779.

Sheline Y, Sanghavi M, Mintun M, Gado M (1999): Depression duration but not age predicts hippocampal volume loss in medical healthy women with recurrent major depression. *J Neurosci* 19:5034–5043.

Sheline Y, Wang P, Gado M, Csernansky J, Vannier M (1996): Hippocampal atrophy in recurrent major depression. *Proc Natl Acad Sci U S A* 93:3908–3913.

Stein-Behrens B, Elliott E, Miller C, Schilling J, Newcombe R, Sapolsky R (1992): Glucocorticoids exacerbate kainic acid-induced extracellular accumulation of excitatory amino acids in the rat hippocampus. *J Neurochem* 58:1730–1735.

Stein-Behrens B, Lin W, Sapolsky R (1994a): Physiological elevations of glucocorticoids potentiate glutamate accumulation in the hippocampus. *J Neurochem* 63:596–602.

Stein-Behrens B, Mattson M, Chang I, Yeh M, Sapolsky R (1994b): Stress exacerbates neuron loss and cytoskeletal pathology in the hippocampus. *J Neurosci* 14:5373–5380.

Tombaugh G, Sapolsky R (1993): Evolving concepts about the role of acidosis in ischemic neuropathology. *J Neurochem* 6l:793–803.

Tombaugh GC, Sapolsky R (1992): Corticosterone accelerates hypoxia-induced ATP loss in cultured hippocampal astrocytes. *Brain Res* 588:154–158.

Traynelis S, Cull-Candy S (1990): Proton inhibition of NMDA receptors in cerebellar neurons. *Nature* 345:347–350.

Turski L, Turski W (1993): Towards an understanding of the role of glutamate in neurodegenerative disorders: Energy metabolism and neuropathology. *Experientia* 49:1064–1072.

Vannucci S, Maher F, Simpson I, Lee W (1995): Effects of cerebral hypoxia-ischemia on glucose transporter expression in immature rat brain. *J Cereb Blood Flow Metab* 15:S287.

Vass K, Welch W, Nowak T (1988): Localization of 70 kDa stress protein induction in gerbil brain after ischemia. *Acta Neuropathol (Berl)* 77:128–135.

Virgin C, Ha T, Packan D, Tombaugh G, Yang S, Horner H, Sapolsky R (1991): Glucocorticoids inhibit glucose transport and glutamate uptake in hippocampal astrocytes: Implications for glucocorticoid neurotoxicity. *J Neurochem* 57:1422–1428.

Volterra A, Tritti D, Tromba C, Floridi S, Racagni G (1994): Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. *J Neurosci* 14:2924–2932.

Whiteford H, Peabody C, Csernansky J, Warner M, Berger P (1987): Elevated baseline and postdexamethasone cortisol levels. A reflection of severity or endogeneity? *J Affect Disord* 12:199–202.

Yatsugi S, Takahashi M, Kawasaki-Yatsugi S, Koshiya K, Sakamoto S, Uematsu D, Shimizu-Sasamata M (1996): Neuroprotective effect of YM90K, a novel AMPA/kainate receptor antagonist, in focal cerebral ischemia in cats. *J Cereb Blood Flow Metab* 16:959–966.

Yehuda R, Southwick S, Nussbaum E, Giller E, Mason J (1991): Low urinary cortisol in PTSD. *J Nerv Ment Dis* 178:366–369.

Yenari M, Fink S, Sun G, Chang L, Patel M, Kunis D, et al (1998): Gene therapy with hsp72 is neuroprotective in rat models of stroke and epilepsy. *Ann Neurol* 44:584–591.

Young D, Dragunow M (1994): Status epilepticus may be caused by loss of adenosine anticonvulsant mechanisms. *Neuroscience* 58:245–261.