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Series Preface

This Series is a joint venture between the International Neuroendocrine Federation and Wiley-Blackwell. The broad aim of the Series is to provide established researchers, trainees and students with authoritative up-to-date accounts of the present state of knowledge, and prospects for the future across a range of topics in the burgeoning field of neuroendocrinology. The Series is aimed at a wide audience as neuroendocrinology integrates neuroscience and endocrinology. We define neuroendocrinology as study of the control of endocrine function by the brain and the actions of hormones on the brain. It encompasses study of normal and abnormal function, and the developmental origins of disease. It includes study of the neural networks in the brain that regulate and form neuroendocrine systems. It includes study of behaviors and mental states that are influenced or regulated by hormones. It necessarily includes understanding and study of peripheral physiological systems that are regulated by neuroendocrine mechanisms. Clearly, neuroendocrinology embraces many current issues of concern to human health and well-being, but research on these issues necessitates reductionist animal models.

Contemporary research in neuroendocrinology involves use of a wide range of techniques and technologies, from subcellular to systems and whole organism level. A particular aim of the Series is to provide expert advice and discussion about experimental or study protocols in research in neuroendocrinology, and to further advance the field by giving information and advice about novel techniques, technologies and interdisciplinary approaches.

To achieve our aims each book is on a particular theme in neuroendocrinology, and for each book we have recruited an editor, or pair of editors, expert in the field, and they have engaged an international team of experts to contribute Chapters in their individual areas of expertise. Their mission was to give an up-date of knowledge and recent discoveries, to discuss new approaches, ‘gold-standard’ protocols, translational possibilities and future prospects. Authors were asked to write for a wide audience, to minimize references, and to consider use of video clips and explanatory text boxes; each Chapter is peer-reviewed, and each book has a Glossary, and a detailed Index. We have been guided by an Advisory
Editorial Board. The Masterclass Series is open-ended: books in preparation include Molecular Neuroendocrinology; Computational Neuroendocrinology; and Neuroendocrinology of Appetite. Feedback and suggestions are welcome.

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Preface

A common feature of various definitions of stress, as experienced by people and animals, is a change in the internal or external environment that threatens homeostasis, in a way that may endanger survival or lead to ill-health. From the earliest formulation of concepts of stress it has been convincingly established that central to automatic physiological coping responses to stressful stimuli (stressors) are the sympathetic–adrenomedullary (SAM) and hypothalamic–pituitary–adrenocortical (HPA) neuroendocrine systems. The rapid responses of these systems liberate into the circulation biologically powerful chemicals, respectively catecholamines and glucocorticoids, and these hormones have bodywide actions to mobilize energy, increase blood flow to essential organs and to optimize brain activity to deal with the emergency. Because these hormones are so powerful, with deleterious actions when high levels are maintained, it is advantageous to restore the stress-induced secretion back to normal basal activity when the danger has passed.

The chapters in this book aim to highlight current knowledge of the organization and physiology of these stress response systems, how the impact of dysregulation of these systems is being investigated and to consider ways in which contributions to both psychiatric and physical diseases as a result of chronic stress effects on HPA axis function, in particular, can be critically addressed in basic research. Chapters have been organized in a sequence that starts with analysis of mechanisms in the key nodes in the adrenomedullary and HPA responses to stress, using animal and human models. The next chapters move on to consider how animal models are being used to address human stress-related disorders, while the final chapters focus on the programming by early-life experiences of HPA axis stress responsiveness and the process of drug design and testing aimed at specific molecular targets important in modulating stress responses.

Chapters 1 and 2 show how it has become clear that the activity of the HPA axis is controlled by multiple interconnected central neural networks and that different types of stressor are processed by anatomically and neurochemically distinct networks, predominantly aminergic or catecholaminergic, converging on to corticotropin releasing factor/hormone (CRF/CRH neurons in the hypothalamus) and regulating sympathetic
nervous system output. These circuits both provide feedforward control, via inhibitory and excitatory inputs to CRF/CRH neurons, and mediate glucocorticoid negative feedback inhibition, which can be rapid via membrane receptors. Plasticity or adaptation of the elements of the HPA axis is a feature of chronic stress, and the synaptic and cellular mechanisms in CRF/CRH neurons, hippocampus and amygdala are being uncovered with electrophysiological techniques (Chapter 3). A similar approach, combined with modelling of electrophysiological properties is revealing details of how secretion of ACTH by anterior pituitary cells is triggered (Chapter 4). Complementary gene expression studies on chromaffin cells in the adrenal medulla reveal profound plasticity in these cells, as a result of even acute as well as chronic stress, which sustains adrenaline production (Chapter 5). In Chapter 6, human studies involving brain imaging and physiological readouts of autonomic responses that have validated animal studies of stress circuitry are detailed; these are providing new insights into the distinct importance of responses of the SAM and HPA axes in the perception of stress.

In Chapter 7, critical discussion and advice about issues, including ethical considerations, that are of key importance in the rational selection of experimental animal models for chronic stress studies, while limitations in interpreting neuroendocrine and behavioural consequences in the context of translation to human disorders are a recurring theme in subsequent chapters. The essential importance of these issues is evident in the animal studies designed to model mood disorders in humans, especially depression, for which chronic stress is evidently a key etiological factor – raising a central question about the role of HPA axis dysfunction in depression. However, lack of verbal communication means that the experimenter must use carefully validated behavioural tests to assess mood in animals; these tests in rodents are described and compared in Chapter 8. Furthermore, the importance is explained of the initial definition of a precise research question, such as whether action of a particular gene, in epigenetic interaction with the environment, underlies a particular phenotype induced by stress, to obtain knowledge that can be translated to humans, by designing equivalent tests in people as well as drug treatments. Coping with stress involves behavioural strategies supported by central actions of stress hormones, and these can involve seeking solace in comfort foods or the use of illicit drugs, with potential serious long-term ill-effects (Chapter 9). Study of the neurobiological basis for these damaging behaviours in the context of dysregulation of the HPA axis has emerged as an important area of research and is discussed in depth. Included in this discussion is the proposal that disruption of the diurnal rhythm of the HPA axis is important in contributing to the development of obesity and the metabolic syndrome (Chapter 10).
The final chapters look at the problem of early-life programming of adult disease through upregulation of HPA axis function and the consequent impact on vulnerability or resilience to stress. Maternal stress during pregnancy can have such programming actions in the fetus, despite the presence of a fetal mechanism (in the placenta) that inactivates maternal glucocorticoid and provides some protection, as shown in gene knock-out studies (Chapter 11). Many studies have focused on the impact of quality of interactions between the mother and infant in lifelong programming. Broadly, these have been on studies of disruption of maternal behaviour by an environmental disturbance that is stressful for the mother and on natural variations between individuals in attention given to the young. Investigations on the neurobiological basis of abnormal behavioural outcomes of early-life environmental stress, and the different rodent models, are reviewed (Chapter 12). Primate and rodent studies of differences in individual mothering are surveyed (Chapter 13) and candidate epigenetic mechanisms of HPA axis programming in the offspring are proposed. The last chapter (Chapter 14) proceeds from the viewpoint that adverse consequences of subtle chronic activation of the HPA axis, for whatever reason, as discussed in the previous chapters, may be moderated by subtly reducing levels of glucocorticoid in target tissues, including the brain, by inhibiting reactivation of glucocorticoid from its inactive metabolite. The pathway to producing such a specific drug (an 11β-hydroxysteroid dehydrogenase enzyme inhibitor), and the difficulties, are explained, with the outcome being a possible new treatment for cognitive decline as in Alzheimer's disease.

We hope that readers will find the chapters in this book to be informative and provocative, and that the findings and ideas discussed will stimulate new research in the important, widely ramifying and exciting field of stress neuroendocrinology.

John Russell and Michael Shipston
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About the Companion Website

This book is accompanied by a companion website:

www.wiley.com/go/russell/stress

The website includes:

• End-of-chapter references and glossary
• Powerpoints of all figures and tables from the book
• Demonstration videos

Words or phrases in **bold** in the text are defined in the Glossary (page 340 in the book, and on the Companion Website).

Chapters 3, 6, 11 and 12 with a video link will include the following logo (🔗) with a direction to see the website for the chapter.
CHAPTER 1

Methods and Approaches to Understand Stress Processing Circuitry

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Photomicrograph demonstrating the GABAergic phenotype of central amygdala (CeA) neurons projecting to the posterior bed nucleus of the stria terminalis (pBST). The retrograde tracer Fluorogold was injected into the pBST and visualized by immunohistochemistry in the cell bodies of CeA neurons (brown). This was followed by in situ hybridization for GAD (glutamate decarboxylase) 65 mRNA (black grains), a marker for the GABAergic neurons. The co-localization of Fluorogold and GAD65 mRNA indicates that projections from the CeA to the pBST produce gamma-aminobutyric acid (GABA) and are inhibitory, putatively leading to disinhibition of the hypothalamic–pituitary–adrenocortical axis.
Chapter 1

1.1 Introduction

Understanding mechanisms of neuroendocrine stress regulation requires the use of anatomical approaches to precisely localize neuronal populations that control physiological output. For example, the activation of the hypothalamo–pituitary–adrenocortical (HPA) axis is mediated in large part by 2000–4000 neurons (corticotropin releasing hormone neurons) located in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN), one of about 10 anatomically distinct subdivisions of this nucleus (Swanson and Sawchenko, 1983). Moreover, recent data suggest that upstream control of the HPA axis may be differentially regulated by subpopulations of neurons located within a defined brain region (e.g. the bed nucleus of the stria terminalis; Choi et al., 2007). Finally, regulatory changes of critical functional importance to HPA axis output can be subsumed by subpopulations of neurochemically distinct neurons within the PVN proper, which can be masked when the nucleus is considered as a unit. For example, vasopressin mRNA is normally expressed at very low levels in the medial parvocellular PVN, but is very abundant in neighbouring magnocellular neurons. When animals are adrenalectomized, glucocorticoid feedback inhibition of parvocellular neurons is lost, whereas magnocellular neurons are unaffected. Thus, despite the 8-fold increase in vasopressin mRNA expression in the parvocellular zone, which is involved in a massive drive of pituitary ACTH release, no net change is observed in whole-PVN vasopressin mRNA content due to dilutional effects of the magnocellular signal. All of these issues highlight the need to study central stress regulatory circuits in their anatomical context.

Understanding the anatomical context of stress processing is critical for both delineating normal homeostatic adaptive processes and those that culminate in stress pathologies, including such diverse diseases as post-traumatic stress disorder (PTSD), depression, cardiovascular disease and the metabolic syndrome. The current chapter is designed to provide an orientation on both tried and true and state-of-the-art approaches to stress circuit study, concentrating on methods used to characterize circuit activation, connectivity and function.

1.2 Assessment of stress activation

Tracing stress-regulatory circuits requires the use of methods to report activation of brain regions under study. In order for a particular set of neurons to regulate stress responses, one assumes that they should be either activated or deactivated during stimulation, be it discrete (acute) or prolonged. In the past, investigators have used measures of glucose
metabolism (2-deoxyglucose autoradiography) or mitochondrial activity (cytochrome oxidase staining) to reveal activated pathways. In recent years, these rather crude methods have been replaced by use of molecular markers that precisely report cellular activation. This section will concentrate on molecular markers, as they afford single-cell resolution with the capacity to be combined with circuit mapping methods.

1.2.1 Markers of acute activation: Fos

The gold standard for mapping acute stress activated neurons employs methods to localize the immediate early gene, \textit{cfos}. The \textit{cfos} gene expression is driven by calcium signalling pathways, which are reliably activated during neuronal stimulation (Morgan and Curran, 1989). Post-stimulus induction of \textit{cfos} gene expression is extremely rapid, due to the fact that transcription is initiated by removal of an arrest signal in the promoter region (Schroder et al., 2013). Moreover, \textit{cfos} mRNA is unstable and rapidly degraded and, similarly, Fos protein has a relatively short half-life. This combination of ubiquitous localization, rapid transcription, rapid degradation and short protein half-life allows for assessment of rapid cellular activation against a background of virtually zero in the unstimulated state. These properties make \textit{cfos} gene and Fos protein detection tools an excellent means by which to visualize activated neurons in the brain. While other immediate early genes (such as \textit{egr1} and \textit{arc}) increase transcription after stressor exposure (Cullinan et al., 1995; Ons et al., 2004) (Table 1.1), to date \textit{cfos} is the only gene that exhibits this ‘on–off’ property and makes it the method of choice for assessment of stress circuit activation in an anatomical context.

Assessment of \textit{cfos} induction can be performed using either \textit{in situ} hybridization for \textit{cfos} mRNA or immunohistochemistry for Fos protein. \textit{In situ} hybridization using radiolabelled probes has the advantage of affording quantitation of the extent of stress-induced \textit{cfos} induction, using densitometric analysis (off X-ray film or phosphorimager screens) or grain count analysis from emulsion dipped sections. Since the \textit{cfos} mRNA is rapidly transcribed and degraded, peak levels are typically observed within 30 minutes of stimulus (stressor) onset and generally return to baseline within 90 minutes to 2 hours. Due to the time lag between transcription, translation and generation of a detectable pool of protein, Fos immunoreactivity typically peaks 90–120 minutes after stimulus onset and returns to baseline within 4 hours (it should be kept in mind that both synthesis and degradation will be subject to modification by stressor duration or intensity, so the above estimates should be considered a general rule of thumb rather than a firm guideline). Quantification of an absolute amount of protein by immunohistochemistry is problematic. However, this method of detection is suitable for analysis of numbers
of activated neurons. Moreover, detection of Fos can be combined with immunohistochemistry for other antigens (dual or triple immunofluorescence) or transgene/knock-in fluorescent markers to phenotype-activated neurons or with fluorescent tracers to identify projections of activated neurons (see Section 1.3 on ‘Tract Tracing’). Immunohistochemistry is also quicker and cheaper than *in situ* hybridization (generally a 2–3 day procedure, with no radioisotope or disposal costs) and is generally the method of choice when information on the extent of induction is not required.

Use of *cfos* as an activational marker has been invaluable in identifying stress-activated pathways. However, its use comes with some caveats. First, one cannot assume that a *cfos* negative cell is not activated. There are numerous examples of cell populations that are electrophysiologically active after stress but do not show *cfos* induction (e.g. the CA1 region of the hippocampus). Second, *cfos* does not lend clear information on cellular inhibition, as neurons that are inhibited do not show a *cfos* signal. Finally, *cfos* is rapidly induced by the initiation of stress and,
consequently, differences in post-excitation shut-off may not be visible as a reduction in the number of Fos (protein)-activated neurons detected by immunohistochemistry (i.e. \textit{cfos} is already turned on by the initial depolarization event and cannot report subsequent inhibition).

### 1.2.2 Markers of acute activation: phosphorylated transcription factors

The relatively recent availability of phosphorylation-specific antibodies has enabled immunohistochemical study of engagement of specific signalling pathways following stress. Examples of several phosphorylated proteins tested in the context of acute stress are noted in Table 1.1. These methods go beyond \textit{cfos} by enabling visualization of activated intracellular cascades downstream of membrane stimulation. For example, use of phosphoCREB antibodies permits analysis of cyclic AMP activation in stress-related cell populations (Kovacs and Sawchenko, 1996); use of phosphoERK can indicate activation of the MAP kinase pathway (Khan \textit{et al.}, 2007), etc. Analysis of these markers is useful in providing more in-depth information on the activation phenotype of labelled neurons. However, phosphorylation events occur very quickly after stimulation (5–15 minutes in some cases) (Kovacs and Sawchenko, 1996) and thus it is important to design studies with short survival times to insure optimal detection. Like Fos immunohistochemistry, phospho-antibody staining can be used in combination with phenotypic markers or tracers to provide detailed cellular signatures of neural activation.

In most brain regions, the extent of acute stress \textit{cfos} induction is reduced following chronic stress exposure. Decreased stress-induced \textit{cfos} expression occurs following heterotypic as well as homotypic chronic stress regimens, suggesting that this is a general property of repeated stress circuit activation. However, a small subset of regions show enhanced acute-stress-associated \textit{cfos} activation following recurrent stress exposure, perhaps reflecting differential engagement or ‘recruitment’ of these regions to chronic stress adaptation or pathology. Consequently, measurement of \textit{cfos} in the context of chronic stress can provide clues as to whether manipulations or disease states shift the normal habituation- or sensitization-induced expression pattern, implying impact on the underlying brain circuitry.

### 1.2.3 Assessing chronic activation: \textit{deltaFosB}

Assessment of chronic circuit activation requires detection of a gene/protein that is not expressed at high levels under basal conditions, but is induced by chronic stress. To date, the only molecule that meets this description is \textit{deltaFosB}, which is a protein in the \textit{cfos} gene family (see McClung \textit{et al.}, 2004). Like \textit{cfos}, the \textit{fosb} gene (and, consequently, the \textit{FosB} protein) is induced by acute stress, albeit with a more
Chapter 1

protracted onset (6 hours to peak protein expression) and offset (12 hours to return to baseline). Following induction, the FosB protein undergoes proteolytic cleavage, resulting in a protein product, deltaFosB, that lacks 101 amino acids at the C-terminus. Fortuitously, deltaFosB is resistant to degradation and accumulates in the cell over repeated exposures. Detection of deltaFosB can thereby report chronic stimulation of neurons. Since it is a truncated protein product, one cannot (easily) make an antibody that specifically recognizes deltaFosB. Hence, to identify deltaFosB, studies typically employ detection of FosB immunoreactivity at intervals greater than 12 hours from the last stimulation, assuming that FosB induced by the stress exposure will be degraded (McClung et al., 2004). Given that one cannot always assume equal degradation rates across regions and stimuli, when using this method it is wise to confirm changes in specific regions by Western blot, if feasible (where deltaFosB can be distinguished from FosB by molecular weight).

The fidelity of deltaFosB as a universal ‘reporter’ of chronic stress-related neuronal activation has not been completely validated. Work from our group suggests that deltaFosB immunoreactivity may not be detected in all cell groups following chronic stress. For example, the hypothalamic paraventricular nucleus does not show significant aggregation of FosB immunoreactivity after chronic stress (Flak et al., 2012), despite clear evidence of transcriptional drive (increased CRH and AVP mRNA expression), cellular hypertrophy and increased HPA axis activation following this stress regimen (Herman et al., 1995). Thus, as was the case with Fos, a ‘negative’ FosB signal needs to be interpreted with caution.

Small animal imaging methodologies (e.g. magnetic resonance imaging (MRI), micro-positron emission tomography (micro-PET)) are rapidly evolving and may soon get to the point where activation of neurocircuits may be assessed using non-terminal and, more importantly, repeated measures procedures. It is likely that these methods will provide the next wave of in situ ‘activity mapping’ in brains of experimental animals.

1.3 Stress circuit connectivity

Establishing the location of activated neurons does not provide connectivity information linking interactions between two or more brain regions. To perform this level of analysis, activity mapping needs to be combined with methods to document neuronal connectivity. Tract tracing is a tried and true method of connecting brain regions, dating back to silver degeneration stains in the 1940s. Combination with activation markers is considerably more recent, making use of compounds that are taken up by neurons and transported to cell bodies or terminals.
1.3.1 Anterograde tracing

Anterograde tracers are compounds that are taken up by cell bodies and transported along axons and terminals, generally providing good visualization of fibres and synaptic boutons (Gerfen and Sawchenko, 1985; see Figure 1.1). Examples include phaseolus vulgaris leucoagglutinin (PHA-L) and biotinylated dextran amine (BDA). Protein transport is visualized either by immunohistochemistry (PHA-L, BDA) or fluorescence, the latter using BDA conjugated to a fluorescent compound, such as Alexa488. Generally, these anterograde tracers are not taken up by nerve terminals and thus are not transported retrogradely. By themselves, anterograde tracers indicate that neurons in the region of injection project to defined anatomical locations, thus connecting stress-related nucleus ‘A’ with downstream nucleus ‘B’. To establish whether targeted neurons in nucleus ‘B’ are activated, triple label immunofluorescence needs to

![Dual tract tracing using retrograde and anterograde tracers, injected in different sites.](image)

Retrograde tracers, such as Fluorogold (FG) or cholera toxin beta subunit (CTB) (green), are injected into the region containing terminal fields of projection neurons. These are taken up by neurons and retrogradely transported to neural cell bodies and dendrites. Anterograde tracers, such as phaseolus vulgaris-leucoagglutinin (PHA-L) or biotinylated dextran amines (BDA) (red), are taken up by neural cell bodies and transported down the axon into terminals. In combination, the two techniques can be used to determine whether neurons projecting to stress-regulatory regions such as the PVN are contacted by neurons in upstream regulatory nuclei, allowing for detection of bisynaptic connections. Note that Fos or FosB immunohistochemistry (indicated as a yellow nuclear stain) can also be applied in these models, allowing one to identify whether retrogradely labelled neurons contacted by upstream neural populations are responsive to acute or chronic stress.
be performed, wherein anterograde tracer is combined with detection of an activational marker (such as Fos or FosB) and either a general marker of cell membranes (e.g. microtubule-associated protein (MAP) 2 or a phenotypic marker that provides good membrane resolution. Triple labelling is required, as Fos and FosB are only present in the nucleus and cannot define cellular boundaries abutted by anterograde tracer. Even in this case, results must be interpreted with caution, as evidence of tracer-labelled boutons in apposition to defined membranes does not indicate a synaptic contact per se. Definitive synaptic interactions can be defined by multilabel electron microscopic methods, which have suitable resolution but are considerably more time and labour intensive.

These histological methods are limited in the information provided about the nature of the neurons projecting to identified targets. Neither PHA-L nor BDA are thought to be selective with regard to phenotype of filled neurons and injections are generally spheroid or ovoid, which does not generally correspond to nuclear boundaries. In addition, spread outside a region of interest is an issue and several injections encompassing a given area and its surround are required for accurate conclusions. To date, these methods are not widely used in mice as, logistically, targeting very small areas of mouse brain can be difficult. To get around these limitations, **anterograde tract tracing** can also be performed using transgenic or knock-in mice that express a fluorescent molecule that can be localized to nerve terminals. Tagged molecules are generally driven by specific promoters, affording the significant advantage of defining projections from particular neuronal phenotypes, provided the reporter molecule is transported. Recent studies using multiple transgenes or knock-ins indicate that the ability to gather multiple pieces of information from the same mouse, e.g. combine expression of the phenotype-specific marker with ion channels capable of activating or inhibiting the neuron using **optogenetics**.

Anterograde tracing can also be assessed in small animals using manganese-enhanced MRI (MEMRI). This method employs local injection of manganese prior to scanning (Canals et al., 2008). Manganese is taken up by neurons (via calcium channels) and is anterogradely transported to presynaptic terminals. The ion is released at the synapse and taken up by postsynaptic neurons, beginning a new cycle of transport. Thus, the transport of manganese across synapses can identify multisynaptic networks. However, the potential toxicity of the ion is a consideration for these studies.

### 1.3.2 Retrograde tracing

Retrograde tracers are compounds that are taken up by nerve terminals and transported back to the cells of origin (e.g. Fluorogold; see Schmued
and Fallon, 1986) (see Figure 1.1). These compounds are generally packaged in endosomes and moved to the cell soma by rapid axonal transport. Most tracers also transport into dendrites, thus providing a fairly nice cellular fill. There are a number of variants used for retrograde tracing, including fluorescence compounds (Fast Blue, stilbene derivatives such as Fluorogold, fluorescent beads and endocytosed proteins, such as the cholera toxin beta subunit (CTB)). They can be easily visualized by either endogenous fluorescence or immunohistochemistry (compounds such as Fluorogold are relatively weak fluors and investigators will commonly use immunohistochemistry to reveal the full extent of a retrograde label). Unlike anterograde tracing, ‘backfilled’ neurons are readily amenable to establishing phenotype and activational status using multiple-label methods, thus telling the nature of neurons in area ‘A’ that project to area ‘B’.

While retrograde tracing is a tried and true method, it is important to note some caveats. First, the specificity of many of these compounds to uptake at terminal fields is not necessarily 100%. For example, there are sporadic reports of uptake of Fluorogold into axons of passage. In addition, injections of retrograde tracer can be toxic, particularly to neurons in the centre of the injection.

Similar to anterograde tracing, injection sites are problematic. For example, injections sites do not reveal the specific phenotype of neurons in the region and typically do not conform to region of interest ‘boundaries’. Consequently, anterograde and retrograde techniques are often used together, with retrograde tracers defining cells in area ‘A’ projecting to a region ‘B’ and anterograde tracers verifying that area ‘A’ indeed projects to specific cell populations in area ‘B’.

As yet, there is no transgenic or knock-in equivalent to anterograde tracing for retrograde tracing of pathways in the context of stress. Some methods exist to provide controlled retrograde tracing, but to date all involve introduction or synthesis of retroviral proteins that eventually kill the infected cell. Cytotoxicity makes it difficult to interpret the meaning of Fos or FosB if the integrity of the cell is compromised.

### 1.3.3 Combined anterograde and retrograde tracing

Anterograde and retrograde tracing are sometimes used in combination to explore trans-synaptic connections between neuroendocrine effector neurons in the PVN and upstream regulatory structures. This is especially relevant to neuroendocrine stress regulation, since numerous stress-regulatory regions do not project directly to PVN CRH neurons. These approaches involve injection of retrograde tracer into the PVN and anterograde tracer into candidate regulatory structures, monitoring where anterograde labels terminals abut or appose somata and dendrites of PVN-projecting
neurons (Figure 1.1). This method was used to document bisynaptic links between the ventral subicular region of the hippocampus and the PVN, implicating regions such as the bed nucleus of the stria terminalis and dorsomedial hypothalamus in control of HPA axis output (Cullinan et al., 1993).

Analogous methods can be used to assess both inputs and outputs of a given region, using concomitant injection of anterograde and retrograde tracers into a putative neuroendocrine regulatory site (Figure 1.2). While individual cell types in the region of injection are hard to specify, using multiple-label immunohistochemistry, one can determine the phenotype and excitation state (e.g. Fos or deltaFosB staining) of regions projecting to and receiving information from a small, defined region of a nucleus of interest.

**Figure 1.2** Dual tract tracing using retrograde and anterograde tracers, concomitantly injected. In this case, retrograde tracer fills neurons projecting to a region of interest (green), while anterograde tracers connect with targets of the region (red). Using a phenotypic marker, the identity of cells in receipt of anterogradely labelled terminals can be determined (magenta), as can that of afferent projections (not shown). Note that co-staining for Fos or FosB (yellow) can indicate whether afferent or efferent targets are stress responsive. A complementary analysis of combined anterograde and retrograde tracing is the identification of reciprocal connectivity. Combined injections of PHA-L and CTB in the infralimbic prefrontal cortex (B) lead to both anterograde tracer-labelled fibres (green) and retrograde tracer-filled cells (red) in the paratenial thalamic nucleus, illustrating corticothalamic reciprocity.
1.4 Lesion, inactivation and stimulation approaches

1.4.1 Lesion studies

The field of stress neurobiology/neuroendocrinology owes a considerable debt to lesion studies, which allow one to test whether removal of a brain region causes increases or decreases in stress reactivity. Lesion approaches have been used since the earliest days of neuroendocrinology. Early studies used either large ablations or electrolytic lesions to destroy specific brain regions or knife cuts to sever their afferent connections. For example, our group showed that lesions of large portions of the hippocampus using suction ablations increased basal HPA axis activity and PVN CRH mRNA expression. The effects of total hippocampal lesions were mimicked by severing fibre outflow from the ventral hippocampal region, specifying the infrahippocampal localization of HPA axis inhibitory effects (Herman et al., 2003). While these gross manipulations provide valuable data, they lack anatomical cellular specificity.

**Electrolytic lesions.** These can be made small enough to specifically target individual nuclei or subnuclei. However, the method involves indiscriminant destruction in the region of the electrode tip, which may compromise fibres of passage and thus cause unintended ‘off-target’ damage to other circuitry. To get around this limitation, most lesion studies now use chemical approaches, generally using excitotoxins such as ibotenic acid. These compounds cause cell death by binding to NMDA and/or AMPA receptors and causing excessive accumulation of intracellular calcium. As these receptors are not present on axons, these agents do not affect the fibre of passage in the region of injection and can generally be considered as cell body-specific.

**Ibotenic acid injections.** These have been used to resolve involvement of relatively small subnuclear cell groups in stress regulation. For example, we used ibotenic acid lesions to demonstrate that the ventral subiculum (but not ventral CA1 or dorsal hippocampus) is involved in inhibiting HPA axis responses to stress. Using small, precisely targeted excitotoxic lesions, we were able to show that the posterior and anteroventral subregions of the bed nucleus of the stria terminalis have differing roles in control of restraint-induced ACTH and corticosterone release (Choi et al., 2007). While useful in generating focal damage, the method shares some of the same concerns mentioned above for tract tracing, in that lesions rarely conform to defined subnuclear boundaries. Consequently it is important to use off-target ‘misses’ to prove regional specificity. In addition, with a few exceptions (e.g. magnocellular PVN neurons) ibotenic acid destroys all neurons in the injection zone and thus cannot define cell phenotypes responsible for cellular actions.
**Phenotype specifying lesion methods.** These can be employed to query stress circuitry. 6-Hydroxydopamine (6-OHDA) is used extensively to demonstrate the role of catecholaminergic systems in stress regulation. This compound causes severe oxidative damage and cell death of noradrenergic, adrenergic and dopaminergic neurons when injected into regions containing cell bodies, axons or terminals. Since uptake systems are specific to catecholaminergic systems, non-catecholaminergic neurons and terminals remain intact. The 6-OHDA lesion method was used to document the stimulatory role of ascending noradrenergic systems on acute HPA axis stress responses (Herman *et al.*, 2003).

**Saporin conjugated antibodies.** These are also used to selectively target specific cell populations. In this case, target molecules (or antibodies directed against target molecules) are conjugated to the ribosomal toxin saporin. By itself, saporin cannot enter the cell and is for the most part harmless. However, if bound to a protein or antibody that is endocytosed, the saporin is introduced into the cell and toxic actions are able to proceed. This method has been used to show the importance of forebrain cholinergic neurons in hippocampal regulation of stress responses (targeting the low affinity nerve growth factor receptor, which is specific for cholinergic neurons) (Helm *et al.*, 2004); verify the contribution of norepinephrine and epinephrine neurons to HPA excitation at the level of the PVN (DBH-saporin) (Ritter *et al.*, 2003); and confirm the role of GABAergic neurons in the bed nucleus of the stria terminalis to acute stress regulation (vGAT-saporin) (Radley *et al.*, 2009).

**Limitations.** There are caveats that accompany all lesions studies and should be borne in mind. First, effects of lesions are commonly tested days to weeks after surgical procedures. Consequently, they afford some degree of compensation by the brain that may conceivably reduce the perceived functional impact of the structure or cause changes in other regions of the brain that could then affect stress endpoints. Second, lesions are almost always accompanied by some degree of astrogliosis or inflammation, which may contribute to the impact of the circuit disruption.

### 1.4.2 Inactivation/activation studies

‘Traditional’ inactivation studies use compounds that cause extensive local inhibition, such as the GABA-A receptor agonist muscimol, sodium channel inhibitors (such as lidocaine) or calcium channel blockers (cobalt). These compounds reduce neural activity in regions of injection and have the advantage of being able to acutely ‘turn off’ a region and test its involvement in stress regulation. For example, inactivation studies (muscimol) have been performed to demonstrate the role of the dorsomedial hypothalamus in inhibition of the PVN (Stotz-Potter *et al.*, 1996), as well