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COMMENTARY

RECEPTOR CHARACTERIZATION AND REGULATION IN INTACT TISSUE PREPARATIONS

PHARMACOLOGICAL IMPLICATIONS

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A ubiquitous form of cell-to-cell communication involves the release and detection of various chemical signals. These chemicals, whether they be hormones or, in the nervous system, neurotransmitters and modulators, bind to highly specific cellular receptor proteins on target cells. Receptor activation by an appropriate ligand transduces the signal to produce a cellular response. Receptors thus provide the first, and in many ways the most crucial, stage of the target cell's responses to stimulation.

Basically, receptor studies fall into four general thematic groups, sometimes with overlapping techniques. The first theme, characterization, attempts to examine the features of the receptor population under study with a view to describing such characteristics and the role(s) they may play in cellular function. Answers to questions about subunit composition and expression, pharmacological identity and receptor specificity, as well as the number and affinity of the binding site are usual goals. The second theme attempts to answer questions about receptor distribution. Clearly, receptors located in intracellular cytosolic or nuclear domains versus membrane domains have quite different cellular functions. Similarly, localization to different parts of a cell, e.g. in neurons, somatic versus dendritic loci, may provide important insights into function. At a cellular level such studies usually employ antibodies raised against specific receptors or receptor subunits, or mRNAs coding for the expression of these subunits. A third class of studies, one related to the first, is directed at understanding how receptors autoregulate their sensitivity in response to the binding of agonists or antagonists. The ability of a cell to regulate its various receptor populations appears to be a key strategy designed to cope with alterations in incoming transmitter signals. Finally, questions are often posed concerning the nature of the signal elicited by the binding of the ligand to the receptor. Hormone receptors may trigger genomic alterations in cell morphology and/or function; neurotransmitter receptor activation may lead directly to the opening of ion channels and alterations in neural activity, or to changes in second messenger activity followed by various secondary and tertiary effects.

The methods by which receptors are studied dictate, in large part, the questions that may be posed. In this article we will consider various methods for the study of receptors and the relative advantages and disadvantages of each. We will also demonstrate that, for at least some of the types of studies mentioned above, the use of "living" cells contained within relatively intact tissues (e.g. 100–700 μm thick tissue slices) offers unique advantages not to be found using more conventional methods.

While recognizing that there is no ideal method for all circumstances, and none that will allow all of the above questions about receptors to be answered, what are the minimum essential characteristics of any method? Most investigators would probably agree that the ability to extract quantitative data as well as day-to-day reliability of an assay are crucial factors. The method employed should also be able to provide accurate characterization of a given receptor population using conventional analytical methods, for example association and dissociation kinetics, saturation binding, and competition studies. To be most useful, the method should be relatively fast and easy to use with a minimum requirement for expensive equipment. Most importantly in our view, the method should enable the study of cell-surface receptors on intact cells contained within a "normal" tissue matrix. This last point may be of particular relevance for understanding the nature of regulation of G-protein-linked receptor populations.

Table 1 lists the various receptor methods presently employed, describing advantages and disadvantages of each. In brief, while there are numerous variations, four radioligand-based methods are in use, the first two the most common: (1) various homogenate/cell fraction assays (membrane, cytosol, nuclear) of differing degrees of purity of the isolated fraction (see Ref. 2 for a general review); (2) *in vitro* autoradiography, usually of thin, previously frozen

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Table 1. Comparison of radioligand receptor assays

Preparation	Advantages	Disadvantages
Homogenate	1. Quantitative with scintillation methods 2. Can control experimental variables (e.g. pH, ionic concentrations, temperature)	1. Unstable in storage 2. Cannot distinguish cell types or receptor distributions 3. Makes assumptions about receptor loci 4. Shows lower binding values (B_{max} and K_d) than intact cell preparations 5. Cannot distinguish surface from internal receptors 6. Requires specialized equipment (e.g. centrifuge, manifold filters)
In vitro	1. Quantitative with film densitometry and scintillation methods 2. Can give regional, sometimes local (cellular) receptor distributions 3. Some control of experimental variables	1. Cannot distinguish cell surface from internal receptors 2. Requires specialized equipment (cryostat, densitometer for film quantification) 3. Autoradiogram film exposure time is variable (days to months)
Intact tissue	1. Quantitative to conventional criteria with scintillation methods 2. Rapid preparation and data acquisition with minimal specialized equipment 3. Compatible with regulation studies 4. May use selective radioligands to distinguish surface from internal receptors 5. Allows receptors to be studied under physiological conditions, but with some control over experimental variables 6. Makes no assumptions about receptor distributions at local or cellular levels 7. Shows generally higher B_{max} values (sometimes also K_d) for most receptor populations 8. May avoid the uncontrolled release of degradative or regulatory enzymes from fractured cells 9. Can be used in conjunction with homogenate of *in vitro* autoradiographic methods	1. Control of experimental variables more restricted 2. Efficacy of agonist action may be restricted by active uptake mechanisms or degradative enzymes 3. Some radioligands, notably agonists, cannot be used successfully*
In situ imaging	1. Studies receptor characteristics and function in intact systems	1. Requires much specialized, expensive equipment 2. Presently has limited choice of radioligands 3. Numerous complicating variables intrinsic to *in situ* studies [i.e. lack of control of virtually all experimental variables (e.g. local drug concentrations)]

* See Lanius and Shaw [1] for labelling of kainate receptors in living cortex slices with [^{3}H]kainate.

tissue sections [3]; (3) a living slice preparation in which groups of intact cells exist in a "normal" cytoarchitectural/ionic milieu and, in the case of neural tissue, in relatively intact circuits [4–6]; in some ways such slice methods are related to *in vivo* radiolabelling methods (see also below); and (4) *in situ* imaging methods [7]. In the following, we will consider these methods, especially the intact slice method, for studies involving the use of radioligands. In some cases, our discussion will also apply to the use of various fluorescent ligands. We will not discuss antibody or biotin labelling of receptors or the labelling of mRNAs for receptor subunits since these latter methods almost always employ fixed cells in either cultures, sections, or slices, and are mainly intended as a means to visualize receptor distributions.

Origins of the tissue slice binding assay

The now classical homogenate receptor assay continues to provide a vast amount of information on drug/neurotransmitter interaction at receptors (or binding sites) located on various cellular elements [2, 8]. This assay has revolutionized the study of neuroscience and provided data which, at the time, were unobtainable by any other technique. However,

evidence has now accumulated showing that there are quantitative and qualitative differences between receptors in intact cells and those in broken cell preparations [9–14]. Such studies question the physiological validity of a preparation which examines receptors forcibly removed from their natural cellular environment. This is not to imply that the investigation of isolated receptors is of no value, merely that the study of receptor-related physiological, and even pathological, events may better be performed in intact tissue.

The most direct method might thus appear to be an *in vivo* approach. In practical terms, however, an *in vivo* paradigm is severely compromised through a combination of cost (the need for large amounts of labelled compounds) and, for the brain, the difficulties of drug penetration of the blood–brain barrier. An alternative method was suggested by experiments in tumour cell lines and in primary cultures, which provided new insights into agonist–receptor interactions (e.g. β-adrenergic receptors [15]; opioid receptors [16]). Freshly disaggregated cells have also been studied successfully in radioligand assays (brain [17–19]; heart cells [20, 21]). In the latter cases, cell viability exceeded 90% and the receptor binding data were often impressive, although it must be kept in mind that disaggregated cells are in some ways damaged. Neurons are obtained via a mechanical sieving technique that almost certainly severs axons and dendritic trees; cardiomyocyte cell membranes may be affected by the collagenase used to disaggregate the tissue. Nevertheless, such experiments on intact cells represented a critical step towards receptor binding in physiologically relevant preparations and raised the potential for providing a system in which a biological response might be correlated with receptor assays in the same cells. We have developed a complementary approach in which receptors are examined on intact cells contained within tissue slices. This technique, initially designed to study receptors in the brain, uses relatively thin sections (100–700 μm; 300–400 μm is typical in our experiments) containing living cells capable of being maintained for many hours *in vitro*. The use of brain slices in neurobiological, mostly electrophysiological experiments, has received wide attention [22–25] and there is no doubt that this is a powerful and versatile preparation.

We recently reviewed the literature concerned with binding assays using tissue slices, including brain and muscle [4, 5]. For a variety of radioligands, binding is reversible, stereospecific, saturable, displaceable by appropriate drugs and is of high (nanomolar) affinity. The methods developed by us are inexpensive, rapid, involve a minimum amount of tissue preparation, and are so straightforward as to allow many workers to enter this field who would not otherwise do so. The techniques as originally employed provided opportunities for the study of cell surface receptors in fresh, non-neoplastic, mammalian brain tissue. In the sections which follow, we also briefly review more recent studies on a variety of receptors in brain, retina, and heart and, in addition, describe a modification of the slice assay which utilizes micropunches cut from tissue slices.

The intact slice method: Advantages and disadvantages

As summarized in Table 1, receptor binding in intact tissue slices offers the following advantages over homogenate or *in vitro* autoradiographic techniques: (1) The cells are in almost physiological conditions and, therefore, are able to express a more complete range of functional behaviour, including receptor regulation. Binding assays can be performed directly after drug treatments, which for regulation studies is important so that re-regulation does not occur; (2) No *a priori* assumptions are made about the cellular/subcellular regions in which the receptors are located, e.g. dendrites vs somata or membrane vs cytosolic or nuclear fractions. Further, the use of hydrophilic vs lipophilic radioligands allows, in principle, surface vs total cell receptor ratios to be determined, a potentially important piece of information for receptors regulated, in part, by sequestration; (3) The use of intact cells minimizes the release of degradative or regulatory enzymes; (4) B_{max} values tend to be significantly higher, likely reflecting true *in situ* values; an apparent increase in K_d values in many cases may be the consequence of endogenous neurotransmitters, again as in the *in situ* condition; and (5) Tissue preparation is relatively rapid, requiring little in the way of specialized equipment.

Along with the advantages listed above, there are some potential disadvantages: (1) Not all radioligands can be used, particularly those whose dissociation rate constants are faster than the time required to rinse out free ligand, as well as those that are lipophilic and cannot be washed out easily. In general, antagonists appear to work best, although we have had success with certain agonists as well [1, 5]. Ligands that are hydrophilic [for example, CGP-12177 (β-adrenergic) or *N*-methyl-scopolamine (muscarinic)] are clearly superior to lipid-soluble ligands such as dihydroalprenolol, which cannot be washed out of slices; (2) Radioligand binding occasionally must be performed at 0–4° to limit the activation of uptake systems. Often a lower temperature is employed for regulation studies to prevent a re-regulation of the receptors, which may occur spontaneously at physiological temperatures. The lower temperature, together with the thickness of the tissue, gives a slower rate of diffusion into the slice and may increase apparent rate constants. However, other than the fact that this tends to make experiments slightly slower, diffusion into and out of the tissue is constant once equilibrium is reached (see Ref. 5 for a discussion at this point) and usually K_d values are not affected significantly; (3) Agonist treatment is often relatively ineffective (though this is also true in intact, dissociated cell preparations; see, for example, Refs. 17 and 20), possibly due to enzymatic degradation and/or uptake at physiological temperatures. Not only may such degradation prevent an accurate determination of agonist action, but it also prevents a precise estimate of agonist concentration over time. In neural tissue, the problem is most acute for putative neurotransmitters,

Table 2. Receptor populations studied using intact tissue preparations

Preparation	Receptor studied*	Type of study	Reference
Cortical slice (rat)	β-Adrenergic	Characterization and regulation	[35]
Cortical slice (rat)	mAChR	Charact/reg	[6, 26]
Cortical slice (rat)	AMPA	Charact/reg	[29, 32]
Cortical slice (rat)	Kainate	Charact/reg	[1]
Cortical slice (rat/cat)	$GABA_A$/BZ	Charact/reg	[27, 28]
Cortical slice (rat)	Dopamine D_2	Charact/reg	[36]
Hypothalamic micropunch (rat)	μ-Opioid	Charact/reg	[5]
Retina slice (rat)	β-Adrenergic	Charact/reg	[37]
Heart micropunch (rat)	mAChR	Charact/reg	[34]
Heart slice (hamster, guinea pig)	β-Adrenergic	Charact/reg	[38]
Muscle slice (mouse)	β-Adrenergic	Charact/reg	[39, 40]

* See text for radioligands employed.

and less so for various agonist analogues (e.g. GABA vs muscimol or acetylcholine vs carbachol). Although each of these can be a significant problem, we have found that, in principle, most can be overcome by the judicious use of particular radioligands and assay conditions. In our view, the advantages cited above, and the type of data generated, far outweigh any such concerns.

Slice preparation: Methods and viability

The methods for producing and maintaining brain "slices" or "micropunches" for receptor binding studies have been well documented in a series of recent articles [1, 4–6, 26–32]. For brain slices, animals are decapitated and their brains rapidly removed into a modified Dulbecco's buffer [6]. The brains are blocked for the required region(s) (e.g. cortex) and sliced in a manual tissue slicer [33] or with a McIlwain tissue chopper. Cardiac tissue is more difficult to handle as a slice preparation, but we have successfully prepared 350-μm thick heart slices using a Campden Vibroslice (model 752/M; World Precision Instruments Inc., New Haven, CT) [34]. Even better cardiac slices have been obtained using a Krumdieck Tissue Slicer (Alabama Research and Development Corp., Munford, AL), specifically designed to prepare slices of living tissue. Although the slice technique is considerably simpler than conventional homogenate or autoradiography assays, we have further modified the method so as to incorporate micropunches (diameter 1–3 mm), in place of slices, into the assay. This simple additional step provides standardized tissue punches of highly reproducible size, shape and weight, and a concomitant reduction in assay variability. This method has also been applied successfully to binding assays on micropunches from rat cortex and retina (see Refs. 4 and 5 and Table 2), allowing greater anatomical specificity in the study of cells of a particular region or division. In both the slice and micropunch technique, the tissue is transported to tissue culture wells containing a modified Dulbecco's buffer [4, 5].

An important consideration in the use of a "living" slice or punch technique is to ensure that the cells are indeed intact and alive. For slices from rat and cat neocortex, we have employed various methods to establish this point. The evidence for cellular viability derives from several complementary measures (detailed in Ref. 6; see also Refs. 4 and 5). First, we have been able to demonstrate at the electron microscope level that neural membranes remain intact for at least 8 hr at 4°. In addition, a trypan blue exclusion test for living cells reveals that with our methods between 70 and 85% of the cells are alive for this same period [6]. We recently used two fluorescent markers* (Molecular Probes, Inc.) to determine either cell viability (calcein-AM) or death (ethidium-homodimer) in cortex slices. These fluorophores indicate that the majority of the cells in the slice are alive. Microelectrode recordings have been made from our slices and have shown robust neural activity for a number of hours. In relation to this last point, we note that our preparation is essentially similar in most respects to brain slice preparations routinely used for a variety of electrophysiological studies [22–24]. In addition, we have been able to document that 2-deoxyglucose, an indicator of cellular activity, is altered following depolarizing or agonist stimulation at 37°, but not 4°.†

Finally, in a number of studies we have shown that various receptor populations can be regulated by depolarizing stimuli or agonists [26–28, 32, 41, 42]. Since receptor regulation usually requires living cells under physiological conditions (see Ref. 26 for a discussion of this point), we view these results as complementary evidence for cell viability. A parallel argument has been made for brain micropunches [5]. Recent work on cardiac slices has demonstrated that cardiomyocytes also remain viable [43].

Receptor characterization

As detailed above, data from characterization studies using tissue slices/punches must be compatible with other binding methods. There are caveats to

* Pasqualotto BA, Lanius RA and Shaw CA, unpublished data.

† Shaw CA and Van Huizen F, unpublished data.

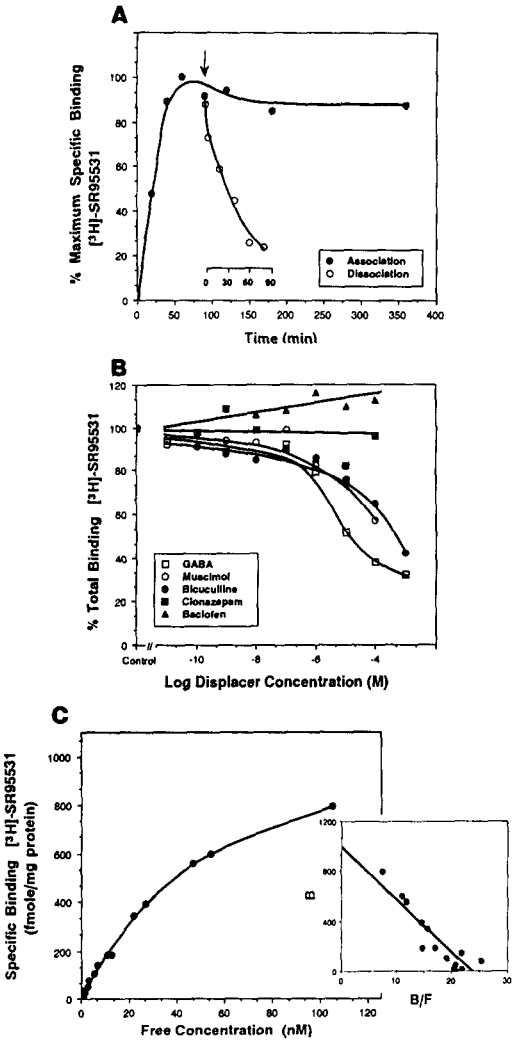

Fig. 1. Characterization of $GABA_A$ receptors in adult rat cortex using living slices. (A–C) $GABA_A$ receptors were studied in cortical slices using the $GABA_A$ antagonist [^{3}H]SR-95531 (see Refs. 27 and 28 for details). Upper panel: association and dissociation rate constant values were determined from time–course experiments; middle panel: competition experiments with various GABA agonists and antagonists; lower panel: saturation binding assay; the inset shows an Eadie–Hofstee plot of these data. The data from the saturation binding experiments gave a B_{max} of 1031 fmol/mg protein and a K_d of 44 nM. Reprinted with permission from *Mol Brain Res* **11**: 273–282, 1991. Copyright (1991) Elsevier Science Publishers B.V. [Ref. 27].

this point which we will discuss in more detail below, but the satisfaction of standard receptor criteria lends support to the utility of the method, especially for comparison with reports using more conventional methods. In Fig. 1, we illustrate a series of characterization experiments for the inhibitory ionotropic $GABA_A$ receptors in rat neocortex using the antagonist [^{3}H]SR-95531 (see Ref. 27 for additional details and citations). Binding is saturable, displaceable, and shows a pharmacological profile consistent with a $GABA_A$ receptor in the central nervous system. Kinetic and saturation binding experiments give K_d values appropriate for the $GABA_A$ receptor. Non-specific binding in the presence of cold SR-95531 is under 15% of total binding for radioligand concentrations used in our regulation experiments.* These data appear to satisfy all conventional criteria for receptor identification and characterization (see Ref. 44). We have also studied a number of other ionotropic excitatory amino acid receptors, as well as various neuromodulatory receptor subtypes in cortex and other brain areas (see Table 2).

The utility of the intact tissue preparation is not limited to brain. We have characterized and quantified a specific M_2-muscarinic receptor in micropunches [1 mm diameter cut from ventricular slices (350 μM)] of rat heart [34]. The use of the water-soluble ligand [^{3}H]*N*-methyl-scopolamine (NMS) allowed us to label M_2 receptors on the cell surface of intact cells contained within the micropunch. Non-specific binding was extremely low (2.8% at 0.27 nM; 7.7% at 3.6 nM) and the specific binding had all the characteristics typical of a cardiac M_2 receptor. Similar studies have now been completed in hamster (Golden Syrian and cardiomyopathic CHF-147) and in dog heart for three distinct receptor types: M_2R ([^{3}H]NMS); βR ([^{3}H]-CGP-12177 [37]; and α_1R ([^{3}H]prazosin).† Most recently, an investigation of the influence of progesterone on M_2 muscarinic receptors in cardiac micropunches showed major differences from results of similar experiments in homogenized heart tissue [34]. In agreement with an earlier report [45], we have shown that progesterone competitively inhibits binding to the cardiac muscarinic receptor. However, in cardiac micropunches the steroid markedly accelerated the dissociation of [^{3}H]NMS from the M_2 receptor, whereas in homogenates the reverse was observed, i.e. no effect on dissociation but a 50% reduction in association rate. This striking difference between broken and intact cells emphasizes again the possibility that the pharmacology of isolated receptors may not truly reflect their physiological properties in intact tissue systems.

In addition to the above, we have used retinal slices and micropunches in a series of studies, which provided quantification of β-adrenergic, M_2-muscarinic, and μ-opioid receptors [37]. The retinal preparation shows even less cellular damage than cortex, with damaged cells restricted to the thin cut edge at the perimeter of the disc. This preparation should also prove of value for studies of receptor regulation in the retina. Recent experiments have demonstrated that neonatal treatment with monosodium glutamate can severely reduce [^{3}H]-NMS and [^{3}H]DAGO binding in retinal micropunches from adult rats‡ in a manner similar to that

* Shaw CA and Scarth BA, unpublished data.

† Wilkinson M, Horackova M and Armour JA, unpublished data.

‡ Wilkinson M, Jacobson W and Wilkinson D, unpublished data.

found in a retinal homogenate preparation [46, 47]. In regard to such experiments, the ability to perform radioligand characterization assays on brain slices without further tissue manipulation allows regulation experiments to be carried out easily and effectively. Examples of such experiments are detailed in the following sections.

Receptor regulation in tissue slices: Response to agonists, antagonists and cellular depolarization

One of the more interesting applications of the intact slice/micropunch receptor binding technique has been to the study of receptor regulation. Much of the definitive earlier work on receptor regulation had been performed in tumour cell lines or in enzymatically dispersed cells. As discussed above, we perceived a clear need to investigate regulatory mechanisms in a physiological brain preparation that retained a considerable degree of tissue and cellular integrity [4–7]. In our earlier experiments we were able to demonstrate isoproterenol-induced down-regulation of β-adrenergic receptors in rat cerebral cortex [35] and more recently have reported agonist-induced down-regulation of μ-opioid receptors in micropunches of rat hypothalamus [5].

Effective regulation studies should allow both the controlled and simultaneous manipulation of large numbers of receptors followed by the ability to halt the process and label the affected receptors before they have a chance to return to their previous state. *In vivo* receptor manipulations often fail this first test due to the presence of diffusion or other barriers. Homogenate or *in vitro* receptor assays fail to provide guarantees for the second condition for the following reasons: Regulation may be very rapid (see Refs. 27 and 28), especially when cellular membranes are fractured to allow the diffusion of regulatory enzymes. Further, fractured membrane preparations may not allow the experimenter to distinguish between receptors that are regulated in the membrane by phosphorylation/dephosphorylation reaction versus those regulated in part by cellular internalization/sequestration.

Examples of more recent regulation studies are illustrated in Fig. 2 for two populations of cortical receptors, the inhibitory ionotropic $GABA_A$ receptor and the G-protein-linked modulatory D_2 dopamine receptor. Here, as in numerous studies by other investigators, receptor regulation appeared to involve a change of receptor number following activation of the receptor by agonist or increases in cellular depolarization (for a brief review, see Ref. 26 for citations). In general, agonists decreased the number of functional receptors and led to a loss of sensitivity to further agonist stimulation (for the latter point see also Ref. 48). Depolarizing stimuli led to either increases or decreases in receptor numbers depending on other variables such as receptor type and postnatal age. $GABA_A$ receptors in adult cortical slices showed a decrease in number following agonist stimulation, but an increase to cellular depolarizing agents [27, 28]. D_2 dopamine receptors appeared to be relatively unaffected by agonists, perhaps due to rapid degradation/uptake of these substances by the cells (see Table 1). Treatment with an antagonist or the depolarizing agent veratridine decreased D_2 dopamine receptor number [36].

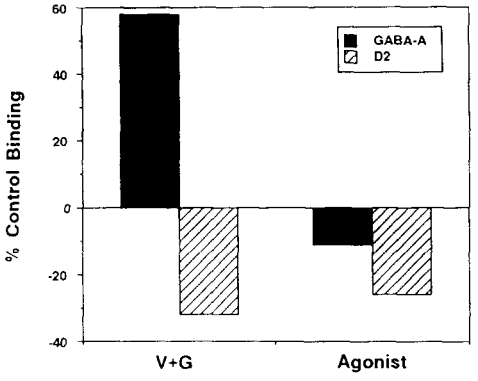

Fig. 2. Regulation of $GABA_A$ and D_2 dopamine receptors in living slices of adult rat cortex. The regulation of the $GABA_A$ receptor was examined following either depolarization with the alkaloid veratridine (in combination with glutamate) or the agonist muscimol. Details of these experiments are provided in Ref. 27. Note the opposite directions of regulation which reflect changes in receptor number. The regulation of the D_2 dopamine receptor was examined following depolarization as above or with the D_2 antagonist sulperide [36]. Note that unlike the $GABA_A$ receptors, D_2 dopamine receptors responded to both types of treatment with a decrease in receptor number. For both receptor populations regulation was induced by treatment with the various agents for 2 hr at 37°. In all cases, differences following treatment were measured as percent difference from control binding values and those shown were statistically significant to at least $P < 0.05$ (Student's *t*-test). Control $GABA_A$ binding at 10 nM, approx. 180 fmol/mg protein. Control D_2 binding at 2 nM, approx. 393 fmol/mg protein.

Receptor regulation in living cortical slices: Age-dependent effects

Experiments designed to explore receptor regulation at different stages in postnatal development have provided the greatest surprise of any of the regulation studies in intact tissues. Examples of such studies are illustrated in Fig. 3 for cortical $GABA_A$ [28] and D_2 dopamine receptors [36]. In both cases, the amount of regulation to agonist or antagonist binding showed significant alterations during development. Perhaps more significantly, the *direction* of regulation was shown to change following depolarizing stimuli and antagonist treatment as a function of postnatal age. At a theoretical level, these may provide supporting evidence that a key aspect of the mechanism inducing neural "plasticity" involves receptor regulation [28–30], and we have noted elsewhere [30] the coincidence between the shift in direction of regulation for various receptor populations and peak period for some forms of neuroplasticity in cortex [49]. At a clinical level, data such as those described above will almost certainly prove to be important for achieving a clearer understanding of various age-related paradoxical drug effects [50]. These two points will be considered in greater detail below.

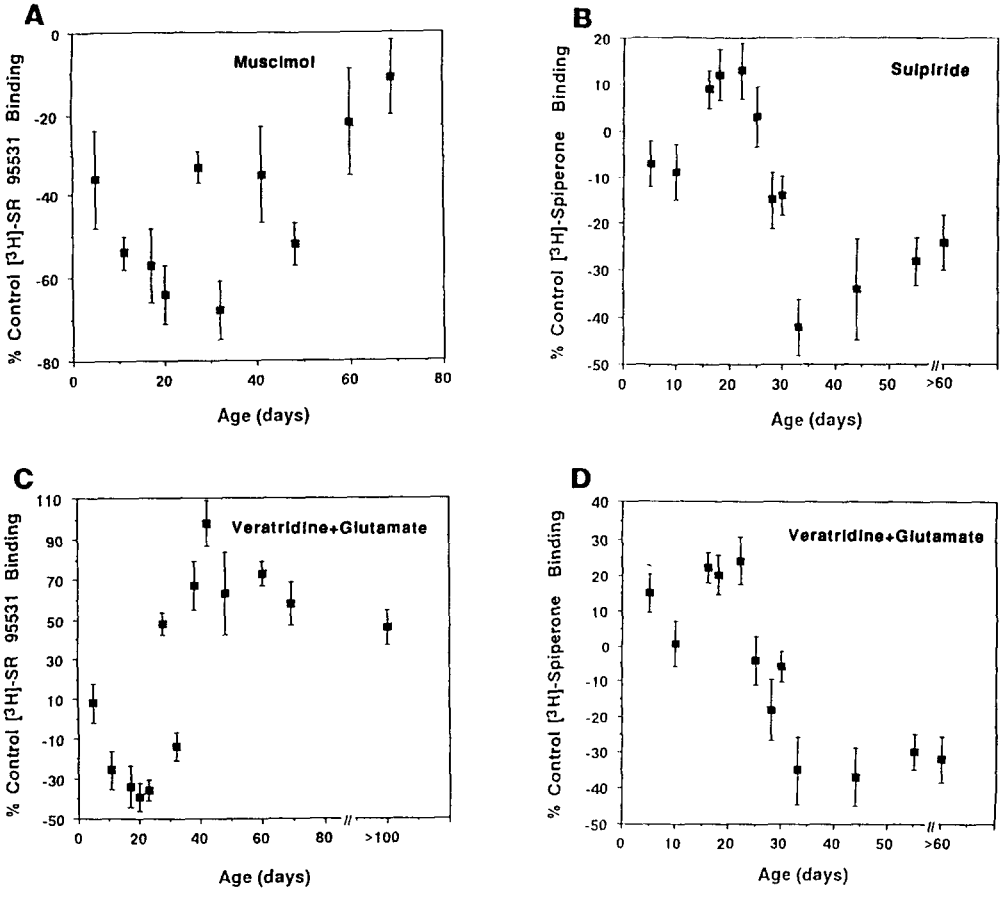

Fig. 3. Age-dependent changes in regulation for $GABA_A$ and D_2 dopamine receptors in living rat cortex slices. Left panels: $GABA_A$ receptor regulation following treatment with the agonist muscimol (A) or veratridine plus glutamate (C) as a function of postnatal age (see Ref. 28). Note the change in direction of regulation from a decrease to an increase in $GABA_A$ receptor number following veratridine treatment versus a change only in amount for agonist stimulation. Right panels: D_2 dopamine receptor regulation following treatment with sulpiride (B), or veratridine plus glutamate (D) (see Ref. 36). In both conditions, the direction of regulation shifted from an increase to a decrease in number during postnatal development. Details of methods and treatment for both receptor populations were as Figs. 1 and 2. Error bars indicate SEM, N = 5–20.

A strategic approach to experiments on receptor function

In relation to the results described above and the implications of these data, we stress that the use of the living slice technique has enabled much more rapid progress than would have been possible using conventional binding methods. Indeed, we believe that in the long-term our ability to provide a relatively simple model of receptor regulation was actually enhanced. This assertion may at first glance seem contradictory given the apparent complexity associated with studying receptor regulation in living cells. However, a brief overview of some of our recent observations on the regulation of ionotropic receptors in the neocortex will make clear the strategic value of this approach. Our initial studies had focused on the effects of "natural" stimuli (agonists and depolarizing agents) to regulate the various cortical receptor populations [1, 26–29, 32]. These studies had shown significant regulation, often with an age dependence. Next, we were able to link the effects of such stimuli with activation of specific kinases and phosphatases [31, 41, 42] (see especially Ref. 42 for citations to primary source receptor phosphorylation literature), and to reproduce effects similar in magnitude and direction with kinase or phosphatase action alone [30, 31]. These latter experiments were performed on frozen/thawed cortex slices since at this stage we wanted to avoid potential secondary effects of membrane potentials and activity-driven neurotransmitter release associated with protein phosphorylation. Our next step was to seek a candidate signal for enzyme activation and this was quickly suggested by reference to our original studies. These studies had shown that various stimuli leading to receptor regulation shared a common feature, namely the ionic currents evoked by receptor activation. Manipulation of selected ionic species in the frozen/thawed slice preparation, in conjunction with the activation of selective kinases

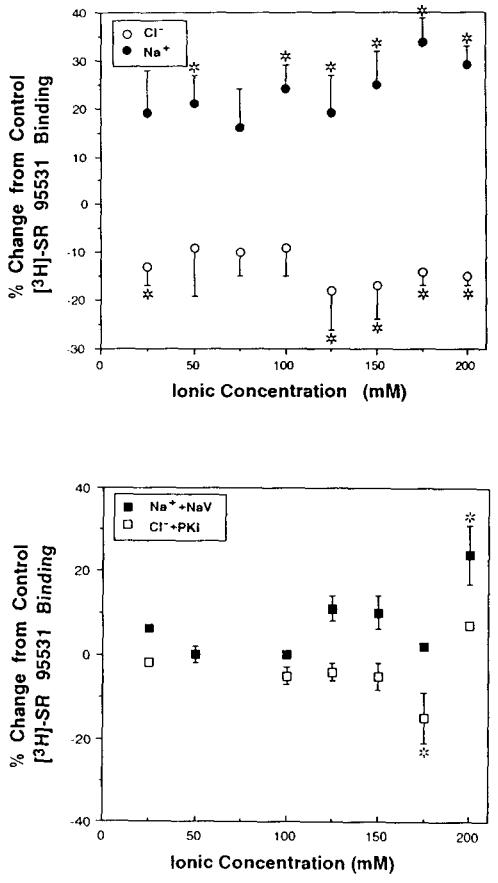

Fig. 4. Ionic dependence of kinase and phosphatase regulation of $GABA_A$ receptors in adult rat cortex. Top panel: Chloride ions (Cl^-) decreased [^{3}H]SR-95531 binding in a concentration-dependent manner; sodium ions (Na^+) increased [^{3}H]SR-95531 binding also dependent on ionic concentration. Bottom panel: Inhibiting endogenous kinase and phosphatase activity using protein kinase A inhibitor (PKI) protein or sodium vanadate (NaV), respectively, prevented the regulation of the receptor in response to altered ionic concentrations (see Ref. 51 for additional details). Significance is indicated by an asterisk; error bars indicate SEM, N = 5–20. Control $GABA_A$ binding at 10 nM, approx. 180 fmol/mg protein.

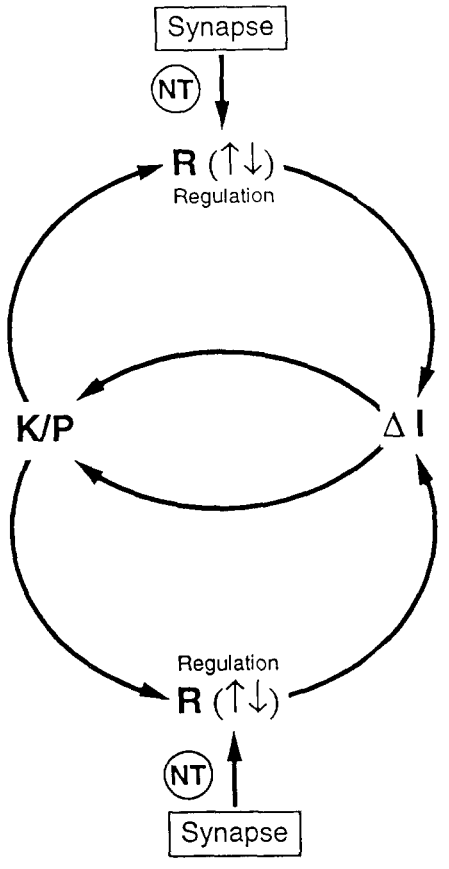

Fig. 5. Proposed molecular cascade for ionotropic receptor regulation. In this scheme, ionotropic receptor activation by neurotransmitter leads to increases in membrane ionic currents. The increase in selective ions stimulates receptor specific kinases (K) or phosphatases (P) leading to a phosphorylation or dephosphorylation, respectively, of specific receptor amino acid residues and a change in the number of functional receptors. The altered number of receptors will subsequent alter the magnitude of the input signal. Note that different receptor populations may be either activated or inactivated by phosphorylation, e.g. AMPA [31] vs NMDA [53]. For $GABA_A$ and AMPA receptors the ions activating kinase activity are Cl^- and Ca^{2+}, respectively (see Fig. 4 and Refs. 51 and 52). Phosphatase activity is stimulated by ionic currents generated through the activation of an "opposing" receptor population.

and phosphatases [51, 52], reproduced all regulatory effects seen with agonists or depolarization [42] (see Fig. 4). Our model of ionotropic receptor regulation is illustrated schematically in Fig. 5.

In essence, we believe that the use of the intact slice preparation has led to a change in our strategic thinking in devising an experimental approach to the study of receptor regulation. Because the slices were alive and studied in close to physiological conditions, we were able to see a full range of phenomena associated with different aspects of regulation. *Only* once we knew that such phenomena existed were we able to modify the technique to provide the same effects under more restricted, essentially reductionist, conditions. In contrast, attempting to proceed in the opposite manner would have required a series of *a priori* assumptions about receptor behaviour, many of which would doubtless have been incorrect. The latter approach is, in fact, the more conventional one, providing the equivalent to a series of snapshot of receptor features, usually out of context to what receptors may actually do in living cells. Our approach recognizes the dynamic interrelated nature of receptor regulation and function and, by allowing for such dynamical processes, has provided a more continuous series of such snapshots, in effect a "video" of the various phenomena. Whether or not one accepts this analogy, it should be readily apparent that our recent

results have provided a wealth of information and a parsimonious model of ionotropic receptor regulation [51].

Slice receptor studies: Implications for pharmacology

As discussed above, intact cell methods for receptor studies, and the results that arise from such methods, may have important implications for pharmacological research at both theoretical and clinical levels. In the first case, our observations of age-dependent receptor regulation and the role of phosphorylating/dephosphorylating enzymes in such regulation have led to the development of a model for some forms of synaptic neuroplasticity [27, 29]. This formulation attempts to simplify the dynamic interactions of the cascade of molecular events that result in receptor regulation. From this we have developed a computer simulation which appears to mimic a form of age-related long-term potentiation (LTP) [54] (for a recent review on LTP, see Ref. 55). Insofar as our interpretation is valid, and given that LTP-like effects are reflective of some types of learning,* our results may suggest pharmacological procedures designed to manipulate and enhance learning and memory.

At a clinical level, the description of age-dependent types of receptor regulation may shed light on various paradoxical drug effects in children [50]. Among the best examples of such effects are the differences in behavioural response to benzodiazepines and tricyclic antidepressants. A number of other drugs may have similar age-dependent effects as well. Prior to clinical trials of novel drugs, intact cell receptor studies in animal models may provide the most economical and practical method to screen for "paradoxical" drug-induced receptor regulation. Finally, we believe that our slice technique can be used successfully as an *ex vivo* binding assay to determine the time course of drug binding following peripheral injection or ingestion. In the past, this powerful method has relied upon receptor binding in homogenates [56], but centrifugation/wash-out steps inevitably induced some degree of receptor–drug association and/or redistribution of bound drug. We have successfully demonstrated that a micropunch assay can provide valuable data on, among other aspects, the time course of opiate antagonist occupation of opioid receptors [5, 57, 58]. This principle could be applied to many drug–tissue interactions *in vivo* and provide important preliminary data for clinical PET scans of receptor binding.

Conclusions

The present article has provided an overview of the development, methods, and utility of an intact cell-tissue slice preparation for studies of receptor characteristics and regulation. As detailed in Table 1, it offers distinct advantages over more conventional techniques in a number of areas, notably expense and the ability to see a range of functional phenomena. It may also be applied to various tissues successfully. Although this technique may also have disadvantages, e.g. the inability to use all conventional radioligands, in our opinion any such disadvantages are minimal. We do not mean to suggest that intact tissue receptor assays can or should replace more conventional assays in all circumstances. Rather, we believe that receptor assays in tissue slices may provide a complementary approach, enabling receptor function to be addressed at a more comprehensive level and one more closely approaching the natural state. The data derived from such studies should prove of value for both theoretical and clinical applications in pharmacology.

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