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Drugs in the brain – cellular imaging with receptor microscopic autoradiography☆

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Abstract

For cell and tissue localization of drugs, receptor microscopic autoradiography is reviewed, including its development history, multiple testing, extensive applications and significant discoveries. This sensitive high-resolution imaging method is based on the use of radiolabeled compounds (esp. tagged with ^{3}H or ^{125}I), preservation through freezing of in vivo localization of tissue constituents, cutting thin frozen sections, and close contact with the recording nuclear emulsion. After extensive testing of the utility of this method, the distribution of radiolabeled compounds has been identified and characterized for estradiol, progestagens, adrenal steroids, thyroid hormone, ecdysteroids, vitamin D, retinoic acid, metabolic indicators glucose and 2-deoxyglucose, as well as extracellular space indicators. Target cells and associated tissues have been characterized with special stains, fluorescing compounds, or combined autoradiography-immunocytochemistry with antibodies to dopamine-beta-hydroxylase, GABA, enkephalin, specific receptor proteins, or other cellular products. Blood-brain barrier and brain entries via capillary endothelium, ependyma, or circumventricular recess organs have been visualized for ^{3}H-dexamethasone, ^{210}Pb lead, and ^{3}H-1,25$(OH)_2$ vitamin D_3. With this *histopharmacologic* approach, cellular details and tissue integrative overviews can be assessed in the same preparation. As a result, information has been gained that would have been difficult or impossible otherwise. Maps of brain drug distribution have been developed and relevant target circuits have been recognized. Examples include the *stria terminalis* that links septal-amygdaloid-thalamic-hypothalamic structures and telencephalic *limbic system* components which extend as the *periventricular autonomic-neuroendocrine ABC (Allocortex-Brainstem-Circuitry) system* into the mid- and hindbrain. Discoveries with radiolabeled substances challenged existing paradigms, engendering new concepts and providing seminal incentives for further research toward understanding drug actions. Most notable are discoveries made during the 1980s with vitamin D in the brain together

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with *over 50 target tissues* that challenged the century-old doctrine of vitamin D's main role as 'the calcitropic hormone', when the new data made it apparent that the main biological function of this multifunctional sunshine hormone rather is maintenance of life and adapting vital functions to the solar environment. In the brain, vitamin D, in close relation to sex and adrenal steroids, participates in the regulation of the secretion of neuro-endocrines, such as, serotonin, dopamine, nerve growth factor, acetyl choline, with importance in prophylaxis and therapy of neuro-psychiatric disorders. Histochemical imaging with high cellular-subcellular resolution is necessary for obtaining detailed information, as this review indicates. New spectrometric methods, like MALDI-MSI, are unlikely to furnish the same information as receptor microautoradiography does, but can provide important correlative molecular information.

Keywords: Estradiol; vitamin D; adrenal steroids; metabolic indicators; lead; blood-brain barrier; amygdala; stria terminalis; circumventricular organs; MALDI-MSI

Contents

1. Introduction

The brain remains a challenge to pharmacologists. Where in the brain do drugs act? Knowing precisely where drugs act is already part of knowing how they act. Do they cross

the blood-brain barrier? Biochemical homogenization and outside scanning procedures frequently fail to provide clarifying information because of tissue heterogeneity, insufficient resolution and sensitivity of methods. Histochemical approaches are needed (Roth and Stumpf, 1969; Stumpf, 2003; 2010). However, conventional methods based on liquid fixation and embedding, while preserving cellular structures, are prone to artifacts due to diffusion and loss of non-covalently bound compounds. New histochemical methods were needed. In order to preserve in vivo sites of drug deposition and achieve high cellular resolution, appropriate freezing procedures had to be developed. Drugs had to be tagged without changing their chemical properties, with signal strength permitting recognition of limited capacity-high specificity sites of receptor binding without obscuring overloading into secondary binding sites and unspecific deposition. Such requirements are not easily met. Instead, convenient and 'expedient' low resolution methods have been favored at the cost of precision and accurate information, including whole-body macroautoradiography, section incubation apposition autoradiography, low-resolution ^{14}C-labeling, and conjugation with large fluorescing molecules (Stumpf and Solomon, 1995). To enable cellular tissue localization of drugs took extensive efforts. Controls, requirements and rules had to be worked out (Roth and Stumpf, 1969; Stumpf, 2003). Dry-mount and thaw-mount autoradiography (Stumpf and Roth, 1966) took several years to establish and test *before* extensive applications during several subsequent decades. Thin section thaw-mount autoradiography - with minor improvements for ease of operation, was renamed 'receptor microscopic autoradiography' (Stumpf, 2003: 2005). Pictorial evidence with discoveries and new concepts garnered over the years testify to the utility of this method (Stumpf and Grant, 1975; Stumpf, 1968a, 1970a, 1995, 1998).

2. Receptor microscopic autoradiography

Treating patients with psychotropic drugs as a clinical psychiatrist and neurologist during the 1950s, I was concerned about unwanted side effects associated with the benefits. There was an apparent need to learn more about sites and mechanisms of drug action. I began to use autoradiography. With conventional fixation-embedding histotechnique, I experienced loss and translocation of radiolabeled compounds (Stumpf et al., 1961). The importance for exploring alternate approaches was also recognized by Sven Ullberg in Sweden, Hilde Levi in Denmark (Levi, 1969) and Lloyd Roth in USA (Roth and Barlow, 1961). In order to work on the development of a method for cellular localization of diffusible compounds, which at the time was considered very difficult or impossible, I took up the invitation of Lloyd Roth to come to the University of Chicago for one year - on a visitor's visa.

Preserving tissue structure for microscopic resolution and, at the same time, retaining drugs and other labile tissue constituents at their in vivo sites of deposition turned out a major obstacle. We learned that 'fixation' does not imply immobilization. Liquid fixatives, as well as embedding media, can produce loss and translocation. Freezing of tissue turned out to be problematic because of ice crystal destruction of cell structure. Ignoring this problem, investigators in academia and pharmaceutical companies resorted to macroautoradiography with thick slices of frozen whole animals. This expedient but low resolution method is applied even nowadays in preclinical ADME studies. While providing convenient surveys

of drug distribution, it does so without discerning specific sites of action from unspecific sites of loss. Lacking cellular resolution, it misses important sites of receptor binding. Such whole body overviews with 'robust quantitative' distribution of radiolabeled compounds agree with equally limited data from conventional cut and burn radioassays. Amazingly, this approach satisfies the requirements of regulatory agencies for the approval of new drugs, but contributes little to the elucidation of drug actions (Monro, 1994; Eichler and Mueller, 1998; Lin, 2006; Stumpf, 2007a). Microautoradiography of diffusible non-covalently bound compounds requires certain precautions for preserving the in vivo positions of labile tissue constituents that include most hormones and drugs. Since conventional liquid fixatives and embedding media cause translocation and loss, both must be excluded for preserving structure. Rapid freezing appeared necessary. During slow freezing of whole animals, as applied in whole-body autoradiography, disruptive ice crystal formation occurs. The size of intra- and extracellular hexagonal ice crystals had to be kept low, adequate freezing temperatures and speeds had to be investigated, and safe temperatures for the storage of tissue samples and frozen sectioning had to be determined (Stumpf and Roth, 1967, 1968). In order to avoid diffusion artifacts during thaw-mounting and wet tissue interaction with nuclear emulsion (chemography), we realized sections needed to be less than 5 μm thin and attachment to nuclear emulsion needed to be quick and without pressure. This methodology requires skills and experience, and the exact conditions of all steps, including photographic exposure, development, and staining must be carefully observed. Through multiple trial and error explorations and control experiments, we were able to develop workable procedures.

Attempts have been made by others (e.g., Pfaff, 1968; Ullberg and Applegren, 1969; Warembourg, 1976, 1985). 'New methods' with an increased propensity for artifacts led to publications of mixtures of results with unrecognized artifacts (Stumpf, 1969; reviewed Stumpf and Pilgrim, 1994). Problems are related to insufficient controls and lack of rigorous testing prior to experimental use, specifically pertaining to critical low temperature conditions during tissue handling, use of thick frozen sections instead of the tested 3-4 μm thin sections, pressure mounting of frozen sections on emulsion-coated slides, use of liquid fixatives, inadequate staining, and others (Stumpf, 2003). The use of methods with unreliable data negatively influenced the utilization of microautoradiography in drug development and brain research.

2.1. Prerequisite tissue freezing and thin frozen sectioning

Vacuum freeze drying of frozen sections (Stumpf and Roth, 1964, 1966) and thin and ultrathin frozen sectioning was explored on the assumption that utmost care was required to ensure preservation of tissue structure and authentic locations of drugs. During these experiments, frozen sectioning at temperatures below −40 °C - assumed then that such low temperatures may have to be observed, albeit stated to be impossible by Pearse (1960) - we experimented and recognized that cutting feasibility is linked to changing conditions between section thickness and knife and specimen temperatures. Frozen sectioning even as low as −105° C succeeded (Stumpf and Roth, 1965). This gave rise to the development of cryo-ultramicrotomy. For light microscopic autoradiography, it turned out, for cutting 4 μm thin frozen sections and preservation of tissue structure, temperatures below – 40° C are not

required. Regarding the dry-mounting method, vacuum freeze-drying frozen sections at low temperatures, and dry-mounting of freeze-dried sections on emulsion-coated slides yielded excellent results, but was demanding. Comparisons of results between the two methods validated those from the less involved thaw-mounting of thin frozen sections. Therefore, the simpler procedure was favored for routine use, leaving the dry-mounting of freeze-dried section as control and for special requirements. This large number of arduous tests over several years – exploring limits and suitable conditions of freezing, low temperature frozen sectioning, and freeze-drying (Stumpf and Roth, 1969), served as a back up of controls and a foundation for the routine 'receptor microscopic autoradiography', which in essence is a frozen section thaw-mounting method (Stumpf, 1998, 2003, 2005).

2.2. Controls for preserving in vivo drug deposition – validation of method

The utility of the autoradiographic method was tested with two diffusible compounds, estradiol and mesobilirubinogen, *before* recommending the method in the literature. In the early 1960s, it was still unclear whether estradiol binds to uterine cell nuclei, cytoplasmic proteins, or plasma membranes. It was surmised that mesobilirubinogen, excreted from the liver, was reabsorbed from the bile. Comparative studies with six different histological methods yielded specific results for each method, with varying degrees of loss and translocation of the diffusible radiolabeled compound during tissue preparation (Stumpf and Roth, 1966).

With conventional fixation-embedding, the mesobilirubinogen test showed radioactivity in the epithelium of bile ducts associated with an empty lumen, otherwise diffused in liver parenchyma, suggesting reabsorption. With our dry-mount and thaw-mount approaches, radioactivity was concentrated in the lumen of bile ducts, with little or no radioactivity in duct epithelium, and radiolabel in the liver parenchyma was apparent in profiles of bile canaliculi, thus arriving at a different conclusion (Stumpf and Lester, 1966). Similarly, with fixation-embedding ^{3}H-estradiol -related radioactivity was diffused throughout the uterus without specific cellular-subcellular concentration, while the dry-mount and thaw-mount methods showed nuclear concentration in muscle, connective tissue and epithelial cells. There was also additional evidence to support the 2-step theory of nuclear action (Jensen et al., 1969). Later we discovered estradiol nuclear uptake and retention in atrial heart muscle, similar to uterine muscle (Stumpf et al., 1977), and in numerous other unexpected targets. Tests of the utility of the dry-mount and thaw-mount methods further included localization of the extracellular space indicators ^{3}H-methoxy-inulin and ^{3}H-mannitol (Brown et al., 1969).

Special staining of the developed autoradiogram permitted further characterization of sites of drug deposition, e.g., methyl green-pyronin for DNA and RNA, or methylen blue-basic fuchsin, all single step stains with differentiation of subcellular structures, in contrast to the common multistep hematoxylin-eosin. Combining immunocytochemistry and microautoradiography in the same preparation, various secretory products and other cytoplasmic components could be localized simultaneously and demonstrated in the autoradiographic image (Narbaitz et al., 1981; Sar et al., 1983). Whenever possible, localization, once accomplished, was followed by quantification through counting of silver grains, now facilitated

with computer programs. Even the number of molecules in a cellular compartment has been calculated after the specific silver grain yield was determined (Stumpf et al., 1981).

2.3. Modifications of method

Is it possible to improve or replace this specific autoradiographic method? Resolution and sensitivity have to be matched, specific localization have to be recognized not only at the cellular-subcellular level but also within its cellular tissue context. Microscopic magnification is necessary. Tritium appears to be an optimal high resolution marker. Replacing nuclear emulsion with another radiation detector and digital recording could potentially simplify the method, but cellular resolution together with tissue orientation would need to be maintained. Nuclear emulsion contributes to sensitivity and resolution. Close contact between the tissue section and radiation detector is necessary. Nuclear emulsion with thaw-mounted sections provides the needed intimate contact for cellular resolution (Stumpf, 2003). 'New methods' have been recommended in the literature but without satisfying the prior testing requirements. With belated recognition of artifacts mixed in with results, these methods have eventually fallen by the wayside, but unfortunately not without damage to the reputation and application of the potentially useful high-resolution receptor microscopic autoradiography.

Improvements must be validated and documented by evidence. Claims should not be made without scientific support. Diffusible test substances with known localizations, like estradiol and mesobilirubinogen used by the author, should be localized on a comparative basis before claims are made. The picture is the evidence. Immunohistochemistry with antibodies to drugs or receptors cannot replace receptor microscopic autoradiography. Drugs not covalently bound will be translocated and lost during incubation. Immunostaining of receptors can be helpful, but vicissitutes have been pointed out, related to variations of the specificity of antibodies, recognition of epitopes, as well as the choice of fixatives, time of fixation, and other treatment steps during tissue preparation (Clemens et al., 1988).

There is always room for improvement and change. The question remains: can the nuclear emulsion thaw-mounting step be replaced with automated digitized recording (Cabello and Wells, 2010)? Digital microautoradiography is a goal for the future. However, currently resolution and sensitivity are lacking. The high resolution and sensitivity of the thaw-mounting method is achieved through intimate contact with the recording nuclear emulsion, a clear image of cells and tissue structure, variable short and long exposure times, and the developed silver grains for quantification. Results of this specific autoradiographic method are still unmatched by any alternate procedures. Chemical imaging methods, like matrix-assisted laser desorption imaging mass spectrometry (MALDI-MSI), and secondary ion mass spectrometric (SIMS) imaging merit further attention (Schwartz and Caprioli, 2010; Touboul et al., 2010; Walch et al., 2008). While these developing methods have the potential of providing specific chemical and spacial information, it is unlikely that they will be able to replace the histochemical microscopic approach, considering the available pictorial evidence. The images generated are specific and unique for each individual method, especially for those generated by mass spectrometry, which are derived from direct molecular measurements (Schwartz and Caprioli, 2010). In order to assess utilities and limitations comparisons

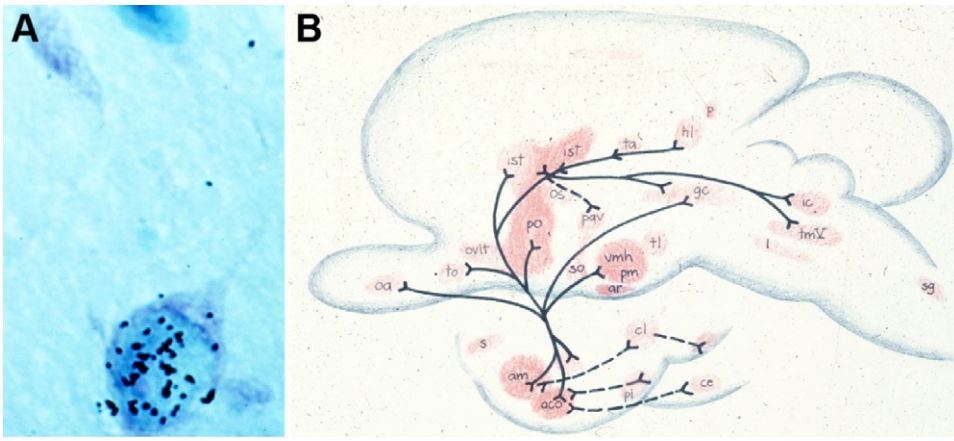

Fig. 1. First demonstrations of cellular (A) and systemic (B) distribution of estradiol in the brain. ^{3}H-estradiol nuclear concentration (silver grains) in neuron of rat infundibular nucleus. Two μm freeze-dried, dry mounted section. At this cellular level, there was distinct nuclear localization, but no evidence for plasma membrane and nucleolar association of estradiol. Accumulation of estradiol concentrating neurons at projection sites of the stria terminalis (B, solid lines), challenging the concept of a 'hypophyseotrophic area' as a circumscribed hypothalamic feedback site (Stumpf, 1968b; 1975). Major accumulations of target neurons: po-nucleus (n.) preopticus medialis, ist-n. interstitialis striae terminalis, vmh-n. ventromedialis hypothalami, pm-n. premammillaris.

between and among methods with identical compounds are needed. As an example, such revealing comparisons have been made *before* the introduction of our dry-mount and thaw-mount methods with two diffusible compounds, estradiol and mesobilirubinogen (Stumpf and Roth, 1966) and later with vitamin D (Stumpf, 2007a) that demonstrated limitations in target identification with radioassays and whole body autoradiography. Value of information and data reliability, much less ease of operation, are to be considered.

2.4. Estradiol in brain and pituitary. Chemoarchitectonic fingerprinting: colocalization with catecholamine fluorescence, antibodies to enzymes and receptor protein. Topographic mapping, phylogeny and ontogeny

First evidence of estradiol neuronal nuclear concentration (Fig. 1A) and selective target circuitry provided our laboratory (Stumpf, 1968b). At that early stage it was immediately recognized that 'estradiol neurons' exist in a pattern that followes the projections of the stria terminalis (Fig. 1B). This did not agree with the concept of the 'hypophyseotrophic area'. With serial section autoradiograms of brain and spinal cord, detailed maps were produced and phylogenetic relationships of representatives of vertebrate phyla studied (Fig. 2) (Stumpf, 1970b; Stumpf and Grant, 1975; Keefer and Stumpf, 1975a, 1975b; Martinez-Vargas et al., 1975, 1976, 1978; Kim et al., 1978, 1979, 1980, 1981; Stumpf et al., 1980a). Using radiolabeled estradiol as a marker, results from ontogenetic and

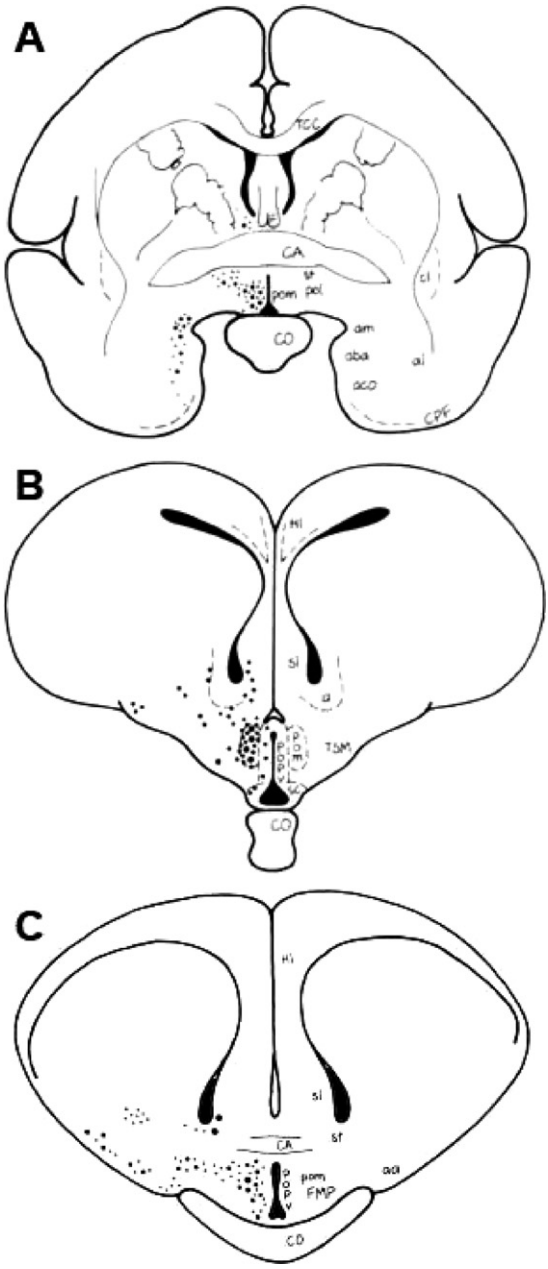

Fig. 2. Estradiol target neuron phylogenetic homology. Target pattern (black dots) in the preoptic-septal -amygdala areas in brains of squirrel monkey (A), ring dove (B), and lizard (C). Schematics from 4 μm cross-section autoradiograms. In early phylogeny, the stria terminalis target system exists as a closely connected unit as visible in dove (B) and lizard (C), segmenting and separating increasingly in mammalian brains as demonstrated in the squirrel monkey (A) (Stumpf et al., 1980a).

phylogenetic experiments demonstrate the original entity and subsequent separation of the preoptic- stria terminalis–amygdaloid unit (Fig. 2) (Stumpf et al., 1978).

In the spinal cord, like in the brain stem, motor neurons did not show estrogen binding under the same conditions, while sensory neurons in the substantia gelatinosa and spinal ganglion, similar to brain stem sensory neurons, concentrated estradiol (Keefer et al., 1973). In contrast, motor neurons are expressive targets of androgen (Reid et al., 1981), corticosterone (Duncan and Stumpf, 1984), and vitamin D (Stumpf et al., 1988), with neurotrophic effects on skeletal muscles. Our studies revealed that there are considerable quantitative differences of receptor binding, with varied nuclear concentration and retention among target cell populations in brain and peripheral tissues. This suggests differential degrees of target activation depending on blood levels. Further high-resolution studies and assessment of relationships to function are needed to understand such action hierarchies, which are apparent for estradiol as well as vitamin D and other substances. Such differences have been studied in the pituitary for different cell types identified with colocalized antibodies (Keefer et al., 1976). The heterogeneous nuclear uptake of ^{3}H-estradiol in pituitary cells is most apparent in autoradiograms at short exposure times (Fig. 3A), but obscured when overexposed (Fig. 3B).

The combination of autoradiography with formaldehyde-induced fluorescence of Falck and Hillarp (Heritage et al., 1977) succeeded in characterizing specific catecholamine neurons as estradiol target neurons (Fig. 4A and B). Colocalization of ^{3}H-estradiol *with catecholamine fluorescence* indicated functional relationships between the estrogenic and catecholaminergic systems that included neurons of raphe nuclei, locus coeruleus, reticular formation (Grant and Stumpf, 1975; Grant et al., 1977; Heritage et al., 1977, 1980). In lieu of formaldehyde-induced fluorescence, antibodies to dopamine-beta-hydroxylase (Fig. 4C) were later used (Sar and Stumpf, 1981, 1983) and confirmed data obtained with catecholamine fluorescence.

In the vicinity of the hypothalamic third ventricle, estradiol concentrating neurons have been colocalized with antibodies to GABA, oxytocin, enkephalin, growth hormone releasing factor, atrial natriuretic factor (Bidmon et al., 1990), and others. The combined localization of radiolabeled compound and antibodies to select proteins opened possibilities for specific chemoarchitectonic fingerprinting of the brain (Stumpf and Jennes, 1984; Stumpf and Sar, 1981a, 1981b). While brain and spinal cord have been the focus, in the same animals many other estradiol targets were discovered, including atrial cardiomyocytes, thymus reticular cells, Leydig cells, prostate epithelium and muscle cells, skin keratinocytes and dermal papillae fibroblasts (Stumpf and Sar, 1976a).

Extensive studies of representatives of vertebrate phyla have pointed to homologous estradiol brain target areas and the significance of estradiol in multiple brain functions, including regions of potential sexual differentiation (Stumpf and Sar, 1978b; Sibug et al., 1991), providing a basis for further detailed investigations.

Because of the wide distribution of estradiol target neurons in the brain, this author pondered that the 2500-year old credo of Alkmaion of Kroton ‘en to encephalon to hegemonicon’ may need to be modified to ‘en to oophoron to hegemonicon’. Not the brain, but rather the ovary, is the leader (Stumpf, 1979). It remains important to note that estradiol conjugated with fluorescein as a marker failed to provide valid data, i.e. to localize nuclear receptors. Attachment of a large molecule to estradiol modified binding properties. Spurious

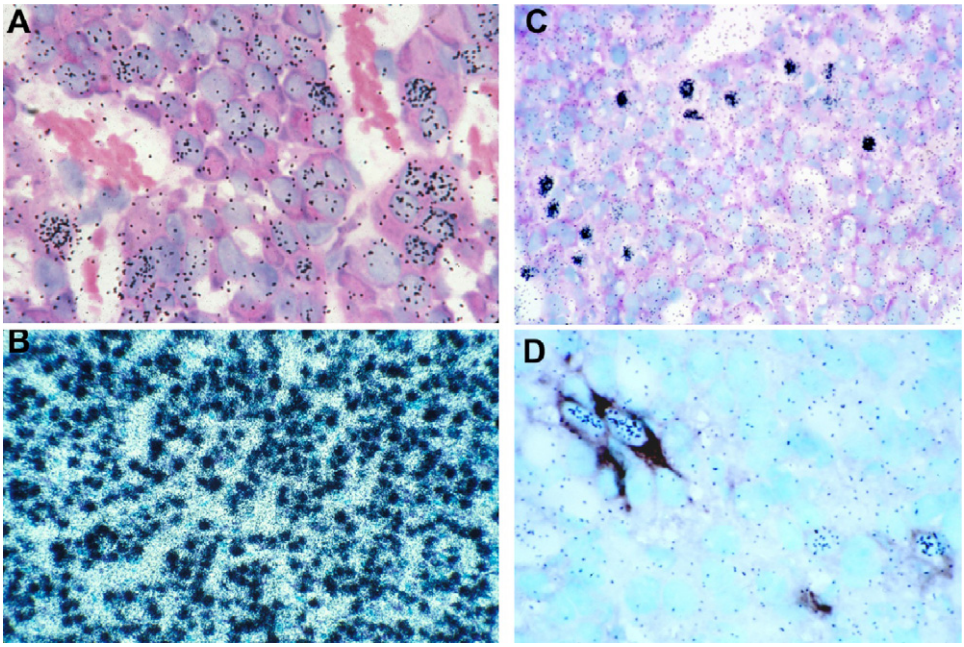

Fig. 3. ^{3}H-estradiol (A and B) and ^{3}H-1,25(OH)$_2$ vitamin D$_3$ (C and D) in rat pituitary autoradiograms, demonstrating the importance of resolution, exposure time, and colocalization. At short exposure rime (A), the differential distribution of estradiol nuclear distribution can be assessed quantitatively. Cells with strongest concentration of radiolabeled hormone are gonadotrophs, recognized through colocalization with antibodies to pituitary hormones, not shown (Keefer et al., 1976). The differenrial cellular concentration is obscured after long (over)exposure, when all cells appear labeled (B). With vitamin D, the differential cellular distribution is still recognizable at long exposure time, with many other cells still weakly labeled or unlabeled (C). The strongly labeled cells have been characterized as thyrotropes after colocalization with antibodies to TSH (D) (Stumpf et al., 1987; Stumpf, 1995)).

results obtained with such conjugates were propagated without prior testing for authenticity (Chamness et al., 1980). It was left to others to provide corrective evidence.

2.5. Progestagens

Whether or not progesterone receptors exist in the brain was long debated and at first denied due to negative data from biochemical homogenates. We provided first clear positive evidence of specific concentration in neurons in various brain regions (Sar and Stumpf, 1973a), indicating again differences in sensitivity and resolution of methods. Cytoplasmic receptor progestin-binding sites were elucidated biochemically much later (MacLusky and McEwen, 1980).

Colocalization of ^{3}H-progestin and antibodies to progestin receptors demonstrated correspondence in the hypothalamic arcuate nucleus as well as in the uterus. In incubation

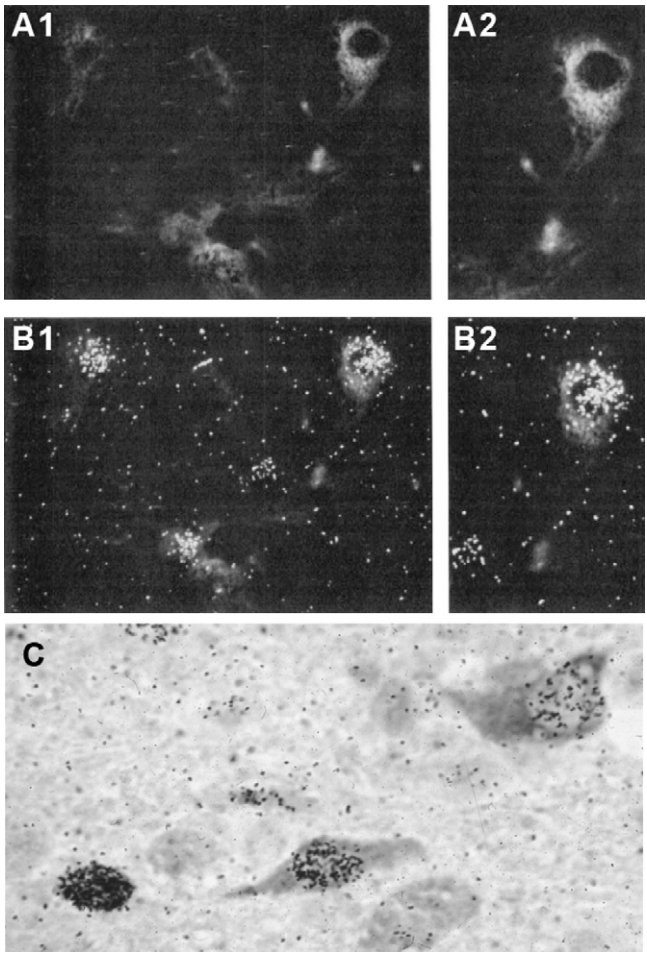

Fig. 4. Combined ^{3}H-estradiol autoradiography. Formaldehyde induced catecholamine fluorescence alone (A1 and A2) and together with ^{3}H-estradiol (B1 and B2). A2 and B2 are high magnification of A1 and B1, showing cytoplasmic fluorescence and nuclear radioactivity in catecholamine neurons in rat nucleus reticularis lateralis (Heritage et al., 1977). Instead of fluorescence, antibodies to dopamine-β-hydroxylase have been used to characterize catecholamine neurons in rat nucleus of the solitary tract, showing cytoplasmic staining of radiolabeled nuclei of two estradiol target neurons, while one labeled neuron (left corner) is not stained (C) (Sar and Stumpf, 1981).

experiments with uterine tissues, it could be demonstrated that receptor protein is present in cell nuclei independent of the presence of steroid hormone, suggesting that receptor translocation from cytoplasm to nucleus does not require binding to hormone (Gasc et al., 1986). With ^{125}I-labeled progesterone, an extensive distribution of nuclear target cells at different developmental ages has been demonstrated in various brain regions, including

cortex and diencephalon (Shughrue et al., 1988, 1992). The distribution of progestin target cell in hamster brain and pituitary after injection of the analog ^{3}H-R5020 has also been studied (Munn et al., 1983). To which degree there is correspondence between progestin and estradiol target neurons and whether or not there are specific sites of progesterone action alone remains to be investigated.

2.6. Testosterone, dihydrotestosterone

Experiments with ^{3}H-testosterone in rats and mice revealed nuclear labeling of neurons in locations in part similar to those of ^{3}H-estradiol, involving the hypothalamic arcuate nucleus, the ventromedial nucleus, medial preoptic and septal nuclei, the bed nucleus of the stria terminalis (Sar and Stumpf, 1972, 1973b; Stumpf and Sar, 1973). Testosterone related distribution occurred throughout the ventromedial nucleus, while the ventrolateral segment was predominant with estradiol. Motor neurons of cranial nerves and spinal cord lamina IX strongly concentrated testosterone-related radioactivity (Sar and Stumpf, 1977; Reid et al., 1975). Combined localization of ^{3}H-dihydrotestosterone and catecholamine formaldehyde fluorescence (Heritage et al., 1981) suggests androgen effects on these neurons in the male similar to estradiol in the female. Testosterone may bind and react as testosterone or as one of its metabolites, dihydrotestosterone or estradiol. This interesting process of local metabolic conversion remains to be explored, aided by correlative localization, e.g. through immunocytochemistry with antibodies to the metabolizing enzymes aromatase and 5-alpha-reductase, to hormone receptors, as well as through in situ hybridization with specific mRNA, and receptor microautoradiography with radiolabeled agonists.

2.7. Adrenal steroids

The extensive presence of corticosterone receptors and strong binding of the hormone in the hippocampus enabled biochemical recognition with excised and homogenized tissues (McEwen et al., 1968). Cellular resolution was, however, required to define the differential distribution in the various cell populations and segments of the hippocampus, dentate gyrus, in other regions of the brain, and spinal cord (Stumpf, 1971; Gerlach and McEwen, 1972). Specific sites of glucocorticoid nuclear receptor binding exist in rat septum, indusium griseum, amygdala, allocortex-periallocortex, i.e., piriform and endorhinal cortex and cingulum, with the highest labeling in the hippocampal area CA2 and indusisium griseum, weak nuclear labeling in the midbrain nucleus ruber (Stumpf and Sar, 1975a) and in catecholamine neurons (Duncan and Stumpf, 1985). Strong nuclear labeling was seen throughout the spinal cord in motor neurons, with lower amounts in neurons of lamina III and VII (Duncan and Stumpf, 1984). Like with other substances, the questions arise: are the patterns of the differential distribution constant, how do they vary qualitatively and quantitatively, and what is the meaning in detail and altogether? The precise localization provides a necessary basis for follow up. Pilot studies with ^{3}H-dexamethasone revealed a different distribution and delayed brain uptake, indicating a blood-brain barrier (Rees et al., 1975; De Kloet et al., 1975; Stumpf et al., 1989a,b). Thirty minutes after the injection, a gradient of radioactivity appeared from the lateral and third ventricle to the surrounding brain parenchyma. At this

time, strong nuclear labeling was visible in peripheral organs but not in the brain. After three hours, radioactivity appeared uniformly distributed throughout the brain with the highest nuclear labeling in cells of the choroid plexus and in neurons and glial cells near ventricles, including neurons of the hypothalamic arcuate and periventricular nuclei, amygdala and hippocampus (Rees et al., 1975; Stumpf et al., 1989a,b). Localization of the mineral corticoid aldosterone in brain regions was first reported by Ermisch and Rühle (1978), applying our thaw-mount autoradiographic method. Further mapping of ^{3}H-aldosterone demonstrated nuclear concentration in neurons of the hippocampus, septum, allocortical regions, brain stem reticular formation, motor nuclei of cranial nerves, and in the meninges. These results suggest that mineralocorticoids have wide ranging effects on different parts of the central nervous system (Birmingham et al., 1979, 1984). The wide distribution of target cells for adrenal steroids in brain and body organs is noteworthy and invites further study.

2.8. Thyroid hormone

Autoradiographic studies with ^{125}I-triiodothyronine in thyroidectomized rats showed prevailing nuclear and additional cytoplasmic concentration of radioactivity in a wide population of neurons and glial cells. A strong cytoplasmic concentration of radioactivity existed in specific regions of the ependyma. Three hours after the injection of radioactive triiodothyronine, strong radiolabeling was seen in select regions of cell bodies and neuropil, e.g., in pyramidal cells of the hippocampus, subiculum, granule cells of the dentate gyrus, Purkinje cells of the cerebellum, neurons of the hypoglossal nucleus (Stumpf and Sar, 1975b, 1978a; Dratman et al., 1982). Controls with radioiodide in thyroidectomized animals did not show the same localized retention and concentration of radioactivity that was obtained with ^{125}I-triiodothyronine. Results of these localization studies are in agreement with biochemical studies and are consistent with known genomic and non genomic effects. They also correspond to widespread actions of thyroid hormone, probably in all cells, potentially in a specific hierarchical pattern. Since ^{125}I can be incorporated into the thyronine molecule in several positions, high specific activity can be achieved, which is favorable for autoradiographic studies. Important further contributions can be expected.

2.9. Vitamin D

First evidence for 1,25$(OH)_2$ vitamin D_3 target cells in brain and spinal cord was provided from our laboratory (Stumpf et al., 1979; 1980).The brain was unexpected and not readily accepted in view of the then current concept of vitamin D's independent peripheral regulation of calcium metabolism that restricted "classical" targets to intestine, kidney, bone, parathyroid, and liver. The fact that bone and calcium metabolism have been studied extensively and other effects slighted or even ignored has given false proof of vitamin D's dominance of calcitropic action. This reflects bias, limited method selection, and narrow perspectives (Stumpf, 1995). In biochemical studies, the brain was repeatedly reported negative for vitamin D (Colston et al., 1980; Gelbard et al., 1980; Sandgren et al., 1991). Even ten years after the histochemical discovery of vitamin D in the brain, "no 1,25-dihydroxyvitamin D3 receptor was detected in cerebrum, cerebellum" with a quantitative

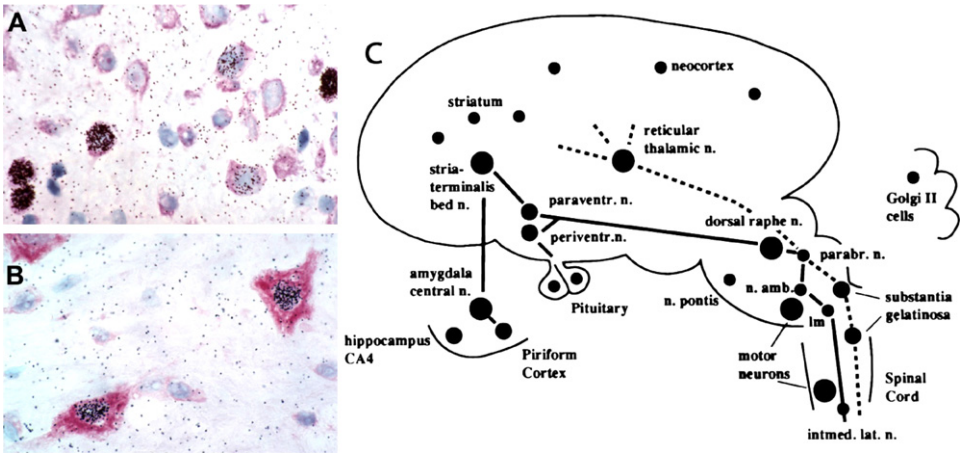

Fig. 5. ^{3}H-vitamin D target neurons in rat central nucleus of amygdala (A) and motor neurons of spinal cord lamina IX (B), representing strong nuclear binding in certain neurons while weak or none in others (Stumpf, 2005). Vitamin D target neuron distribution in the central nervous system (C). The schematic indicates effects on motor, sensory, autonomic-endocrine circuits, and on select cortical, cerebellar, and spinal neurons (Stumpf and Privette, 1991; Stumpf et al., 1988). The size of dots in C reflects different degrees of nuclear binding and suggests selective and differential responses related to vitamin D blood levels.

immunoradiometric assay (Sandgren et al., 1991). In the "evolution of our understanding of vitamin D" (DeLuca, 2008) the brain was not recognized as a target. In the textbook on "Vitamin D" (second edition, Feldman et al., 2005), a chapter on brain (included in the first edition, but restricted to discussion of nerve growth factor) was omitted. And yet, evidence for the existence of specific binding sites in the central nervous system was provided not only as far back as 1979 and 1980 (Stumpf et al., 1979, 1980a), but target neuron populations have been documented and characterized in forebrain, midbrain, hindbrain, and cerebellum of rats and mice (Stumpf et al., 1982; Stumpf and O'Brien, 1987), in the spinal cord (Stumpf et al., 1988), further in brains of hamster (Stumpf et al., 1992a,b,c; Musiol et al., 1992), Zebra finch (Bidmon and Stumpf, 1994a), turtle (Bidmon and Stumpf, 1994b), lizard (Bidmon and Stumpf, 1996), and fish (Bidman et al., 1997), furnishing evidence for both phylogenetic target homology and species-specificity. Homologous target areas include the stria terminalis and central amygdala (Fig. 5A), and motor neurons of cranial and spinal nerves (Fig. 5B).

In some brain regions, nuclear labeling with ^{3}H-1,25(OH)$_2$ vitamin D^3 is equally strong or stronger than in peripheral organs. This supports our concept that systemic calcium regulation is not the predominant role of the sunshine hormone, but rather part of adaptive regulation of growth and repair, reproduction, digestion, defense and immune responses and other vital functions, all supported by vitamin D (Stumpf, 1988b, 1995, 2007b). Since vitamin D research is increasingly expanding beyond the narrow calcium focus, multiple brain targets (Fig. 5C) and related effects have become apparent, as predicted more than

20 years ago (Stumpf and Privette, 1989; Stumpf, 1988a). Autoradiographic data from the 1980s in animals have been supported with histochemical data in the human brain (Eyles et al., 2005). Reports of vitamin D central nervous system actions include effects on brain enzymes and messengers, e.g., cholin acetyltransferase (Sonnenberg et al., 1986), nerve growth factor (Neveu et al., 1994), and serotonin (Privette et al., 1991). Dopaminergic substantia nigra, serotoninergic raphe nuclei and noradrenergic locus coeruleus all contain vitamin D target neurons. Vitamin D effects on tyrosine hydroxylase mRNA in adrenal adrenergic-noradrenergic cells have also been demonstrated (Puchacz et al., 1996). In the anterior and posterior pituitary different cell types are vitamin D targets, predominantly thyrotropes (Sar et al., 1980) (Fig. 3C and 3D), increasing blood levels of TSH and other hormones. Clinical observations about vitamin D effects relate to alterations of mood and cognition, brain development, morbus Alzheimer, multiple sclerosis, depression, schizophrenia, autismus, others (Landsowne et al., 1998; McGrath, 1999; Eyles et al., 2003; 2005; Stewart et al., 2010; Ubbenhorst et al., 2011).

2.10. Retinoic acid

Actions of vitamin A are linked to those of steroid and thyroid hormones. Therefore, knowledge about sites of receptor binding and relationships provide fundamental information. Pilot autoradiographic studies with tritium-labeled retinoic acid in adult mouse brain (Stumpf et al., 1991a,b) provided evidence for nuclear concentration in neurons in distinct brain regions that include caudate-putamen, striatum, bed nucleus of stria terminalis, amygdala central and medial nucleus, piriform cortex, dorso-lateral hypothalamus and arcuate nucleus, olfactory bulb and tubercle, ependymal cells and choroid plexus. In the eye, there was uptake and retention of radiolabeled compound in select layers of the retina and nuclear concentration in certain neurons in the ganglion cell layer. In the anterior pituitary, cells with nuclear and some cytoplasmic accumulation of radioactivity were scattered throughout the gland. While these studies are incomplete, the results provide a promising basis for further studies and invite comparisons with results from experiments with antibodies to retinoic receptors (Krezel et al., 1999; Tafti and Ghyselinck, 2007).

2.11. Ecdysteroids

Ecdysteroids, specifically 20-hydroxyecdysone, are the only endogenous and biologically active steroid hormones in insects (Bidmon et al., 1991a,b). Cellular and subcellular localization of ecdysteroid binding sites became successful with ^{3}H-ponasterone A, an analog with high specific activity of 180 Ci/mmol, using the thaw-mount autoradiographic method (Bidmon and Sliter, 1990). Previous attempts by other investigators remained inconclusive due to low specific activity of radiolabeled ecdysone and 20-hydroxyecdysone. The cellular distribution was mapped after incubation of dissected brains of the fifth larval stadium of Manduca sexta. The autoradiograms exhibited stage-specific differences in the pattern of neurons with detectable binding sites (Bidmon et al., 1991a,b). The results demonstrated that not all cells but only certain cell populations in the larval brain contain ecdysteroid receptors, consistent with expectations that the expression of receptors in

topographically distinct cell populations occurs in a stage-dependent manner during the fifth instar stadium, apparently inducing cell proliferation and/or cell death (Bidmon et al., 1991a,b).

2.12. Glucose and 2-deoxyglucose

Mapping brain functions with metabolic indicators is a valuable diagnostic approach. Experimental imaging with ^{14}C-2-deoxyglucose deposition as an indicator of glucose utilization (Sokoloff et al., 1977) has been widely applied. The traditional use of thick tissue sections with whole-body autoradiography, however, lacks cellular resolution and therefore misses important information (Stumpf, 2002). This and other aspects of the use of ^{14}C-2-deoxyglucose have been investigated (Duncan et al., 1987b; Duncan and Stumpf, 1991). There are regional differences between glucose and 2-deoxyglucose deposition, related to the labeled substrate, the position of the label, and the time selected after administration, as revealed by high resolution autoradiography (Duncan et al., 1987a, 1988; Brüstle et al., 1988). Highest resolution was achieved in our laboratory when dry mounting of 4 μm freeze-dried sections (Stumpf and Roth, 1966) together with thaw-mounting of frozen sections was applied. In addition to accumulations in neuropil regions, high cellular accumulation of radiolabel was recognized in glial cells and in neurons in select regions of the reticular formation of the lower brain stem. Neurons with especially high accumulation of ^{3}H-2-deoxyglucose were located in the paragigantocellular reticular nucleus and in dorsal parts of the reticular formation – under resting conditions at both 5-minute and 45-minute survival periods. These regions are known to be involved in cardiovascular and respiratory regulation (Duncan et al., 1990). A survival time after injection of radiolabeled 2-deoxyglucose of 5-minute instead of 45-minute has been considered as an alternate useful paradigm. However, while 2-deoxyglucose uptake has 'proven to be a versatile tool to study regional brain activation', it cannot be viewed as a 'universal functional activity indicator' (Duncan and Stumpf, 1991).

2.13. 'Blood-brain barrier' (BBB) and ventricle recess (circumventricular) organs

High resolution cellular imaging demonstrates that the 'blood-brain barrier' is not a simple brain barrier, but has certain openings (leaks) related to ependyma and choroid plexus specializations, ventricular recess (circumventricular) organs (Stumpf and Grant, 1975; Ermisch et al., 1992; Rühle, 1992), and possible endothelial transport. Experiments and discussions that do not include in situ microscopic information therefore are incomplete and flawed. Low resolution methods can produce false negatives, as has been the case with quaternary ammonium compounds that showed EEG-changes in the presence of a diagnosed blood-brain barrier, or vitamin D for which brain maps of target neurons for several vertebrate species have been published in contrast to negative evidence from low resolution macroautoradiography and biochemical studies (Pardridge et al., 1985). Assessment of the blood-brain barrier requires high cellular resolution because of BBB-leaks, i.e., possible entry of drugs through ventricular recess (circumventricular) organs, ependyma, and slow capillary penetration. For example, a delayed entry into brain through ventricles has been

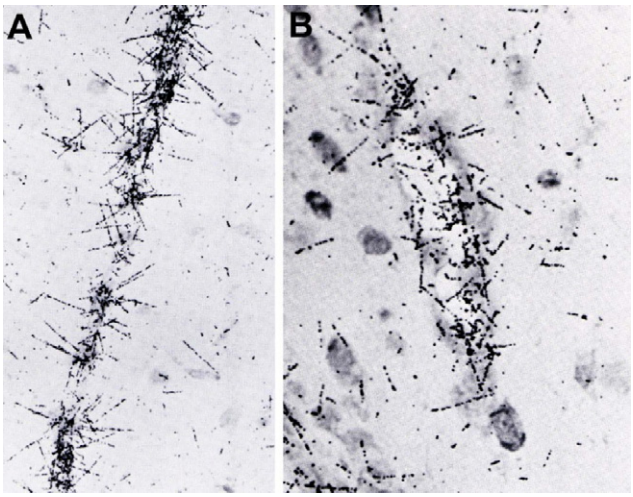

Fig. 6. ^{210}Pb and its decay products concentrated and retained in rat brain capillary endothelium, 24 hours (A, longitudinal section) and 72 hours (B, cross section) after injection. Low radioactivity in neuropil. A similar concentration as in capillary walls existed in choroid epithelium and in circumventricular organs (not shown). The distribution of radioactivity indicates a blood-brain barrier (Stumpf et al., 1980b). Both alpha- and beta particles contribute to the autoradiographic image.

demonstrated with ^{3}H-dexamethasone. At 30 minutes after injection, a gradient was still recognizable from ventricle to the brain parenchyma with neuronal concentration restricted to the periventricular region (Rees et al., 1975). In contrast, in peripheral organs the concentration of radioactivity was several times greater than in the nervous system (DeKloet et al., 1975). With ^{210}Pb lead, by contrast a blood-brain barrier was indicated from autoradiographic pictures that showed concentration of radioactivity in capillary walls (Fig. 6). Neurons remained relatively unlabeled, and even after 72 hours radioactiviity appeared restricted to endothelial cells and circumventricular organs (Stumpf et al., 1980b,c).

3. Discussion

3.1. Histochemistry a via regia to the brain

The history of discoveries of drugs in the brain makes apparent that histochemical approaches have far outperformed biochemical approaches. In the exploration of the brain, the via regia is from histochemistry to biochemistry. Histopharmacology is indispensable in elucidating drug actions (Stumpf, 2010). Target cells frequently are embedded in non-target cells as ‘low capacity-high specificity sites’ and can therefore be missed with biochemical assays of homogenized tissues and low-resolution scans, while ‘high capacity-low specificity sites’ are more easily recognizable, but of less significance and only part of the picture. ‘High specificity-high capacity sites’, accessible to topographic-biochemical approaches

allowed early recognition of estradiol target neuron accumulations in the basal hypothalamus (Eisenfeld and Axelrod, 1965) and of corticosterone in the hippocampus (McEwen et al., 1968). For progesterone, however, there was a gap of several years between the histochemical demonstration of brain localization and biochemically confirmation (Sar and Stumpf, 1973a; MacLusky and McEwen, 1980). For vitamin D, biochemical approaches as well as whole body macroautoradiography failed to recognize specific binding sites, while the high-resolution autoradiography approach provided maps of target cell populations for mammalian and non-mammalian vertebrate species (l.c. above). In all cases, detailed recognition of brain targets required high-resolution microautoradiography (Stumpf and Grant, 1974, 1975; Stumpf and Sar, 1976b; Stumpf, 2003). Immunocytochemistry with antibodies to receptors is helpful and provides additional information, but it does not inform about receptor occupancy and is not quantitative. The review of substances acting in the brain reveals that high resolution is needed for assessing sites of action and that a combined use of low- and high-resolution methods may furnish optimal information. Data from a single method alone, or from several methods with similar deficiencies, remain incomplete and potentially misleading. Complementary information and validation are required, and results from different methods and related perspectives need to be considered (Stumpf, 2007a). Localizing drugs in the brain has been and still is a difficult task. The ability to provide authentic in vivo information with clear cellular morphology in the context of recognizable brain topography is a challenge but achievable through available approaches, including receptor microscopic autoradiography.

False claims based on over-interpretation of low-resolution data and artifacts have undermined the value and importance of tested microautoradiography methods. In developing a suitable method for in vivo tissue and cellular localization, several steps had to be carefully explored. Along the way, valuable discoveries have been made, such as freezing of tissue with no or minimal ice-crystal damage, cutting of thin frozen sections at low and ultra-low temperatures, developing of a portable cryo-sorption pump for freeze-drying of tissue, introducing one-step staining methods to replace the conventional multi-step and less differentiating hematoxylin-eosin, combining autoradiography with immunocytochemistry, introducing effectice controls, introducing quantitative evaluation through silver grain counting and converting silver grains into numbers of molecules (Stumpf et al., 1981; Stumpf, 2003).

3.2. Combining low- and high-resolution imaging perspectives

'Drugs in the brain' remains an important future topic. Localization is a necessary first step in the exploration of action. Localization must contain authentic in vivo detailed information, relevant for meaningful modeling, projection and follow up. Even in its limited scope, this report provides evidence of the utility of high-resolution imaging of in vivo deposition of substances and clarifies that much of this information is unique and unobtainable otherwise. As repeatedly stated: 'the picture is the evidence'. The author of this article is baffled at how pharmaceutical companies release drugs knowing little or nothing about their comprehensive in vivo targets and target kinetics and appear satisfied with mere extrapolations from blood bioavailability, in vitro tests, radioassays of excised organ pieces,

whole body macroautoradiography, and computer models (Monro, 1994; Stumpf, 2007a). How much do we know about the many targets of such common drugs as cardiac glycosides, aspirin, melatonin? Only recently have vitamin D calcium experts come to accept histochemical findings of brain targets of vitamin D published 20 to 30 years ago (Narbaitz et al., 1981). The understanding of vitamin D was changed drastically by new perspectives that were added through histochemical evidence. Specific methods with the confines of their limited results provide nothing more than limited perspectives. Epistemically, different perspectives need to be integrated to enable a holistic view - as made apparent in Chuang Tsu's parable:

> Jo of the North Sea said, "You can't discuss the ocean with a well frog - he's limited by the space he lives in. You can't discuss ice with a summer insect - he's bound to a single season. You can't discuss the Way with a cramped scholar - he's shackled by his doctrines." (Watson, 1964).

Orthodoxy arises when restricted views are developed into dogma by powerful advocates. The calcium dogma of vitamin D has reigned for almost a century. Although increasingly eroded, it is still dominant in textbooks. Findings with our histochemical approach and the related new concepts, initially ignored, followed by only a few, much later attracted an increasing number, although then often without admitting or realizing the origin. – as is the fate of challenging discoveries (Kuhn, 1962; Stumpf, 1980).

3.3. Future applications and development: spectrometric methods, MALDI-SMI

Potential uses of our autoradiography method may include any study that requires topographic information with cellular resolution and elucidation of in vivo localization of diffusible substances, for instance:

- Sites of action and deposition of drugs and analogs as part of ADME preclinical drug development
- Part of comprehensive imaging, providing high resolution information, complementing and validating low-resolution in vivo imaging data
- A basis for functional, biochemical, clinical follow-up
- Maps for metabolic indicators related to specific conditions and diseases
- Characterization (qualitative and quantitative) of sites of action of specific neuroendocrine substances and hormones (e.g., serotonin, melatonin, vitamin D, leptin)
- Characterization of lead compounds, analogues, antagonists
- Follow-up on receptor knock-out mice and study of animal disease models
- Age-related changes: development and aging
- Dose-related changes of receptor binding (micro-dosing, hormesis)
- Correlation, validation, and complementation of methods, e.g., in vitro, in silico, low-resolution imaging, radioassays, whole-body autoradiography
- Target tissue pharmacokinetics, assessment of changing receptor occupation, development of computer programs
- Drug delivery routes and tissue barriers
- Assessment of nanoparticle-mediated drug delivery

- Correlation between receptor protein (immunohistochemistry) and ligand binding (autoradiography)

For high resolution drug distribution studies, at this time no alternate procedure is available, considering the results that can be obtained with the autoradiographic method reviewed in this article. Efforts to replace high resolution receptor microautoradiography with other methods have not been successful, except in a few very limited applications. Over the years, in vivo scanning methods continued to improve resolution in molecular imaging with Micro PET and SPECT (Phelps, 2000), used as independent and combined procedures. Progress has been made with Advanced MRI (Jackson et al., 2008) and Secondary Ion Mass Spectometry (SIMS) (Wu et al., 2010). Spectrometric methods have provided promising data (Walch et al., 2008; Touboul et al., 2010; Schwartz and Caprioli, 2010; Rauser et al., 2010; Ji et al., 2010). For MALDI-MSI, no tagging of molecules is required. Whether spectrometric approaches can be developed into practical, general procedures for drug distribution studies (Reyzer et al., 2010) or whether their utility will continue to be limited to specific applications, remains to be tested and validated with compounds for which cellular-subcellular and tissue distribution patterns have been established, such as, estradiol and vitamin D.

With *MALDI sprectrometric imaging*, molecular finger printing of macromolecular lipids, polypeptides will be of diagnostic value. Spacial localization of covalently bound substances, including certain drugs, is also possible. Such compounds can be immobilized through fixatives at their in vivo sites, and tissue can be treated without loss and translocation. However imaging of diffusible compounds requires special precautions. Fixation, embedding, any liquid phase must be excluded in order to avoid loss and translocation artifacts. Spectrometric methods would require precautions in tissue handling as developed for receptor microautoradiography (Stumpf, 2003). In order to achieve cellular-subcellular tissue resolution of diffusible compounds, which includes most of the drugs, adjustment of procedure is required. Current development and evidence suggest that certain limitations of spectrometric methods - similar to those associated with whole body autoradiography and biochemical radioassays - will be difficult or impossible to overcome and that results of different histochemical approaches will need to be correlated and complement each other. *Immunocytochemical localization* of antibodies to receptor protein has been attempted for the identification of drug receptor target binding sites to substitute autoradiography. Localization of antibodies to receptor protein can provide information identical with that of drug binding, however, results may not always correspond to those of drug binding and specific deposition. Types of antibodies, tissue fixation, accessibility of epitopes, varying composition of receptor protein all can influence results. Comparative studies are needed between receptor microautoradiography and immunocytochemistry. Can microautoradiography be improved? Digitized recording of beta-emissions from frozen or freeze-dried sections in lieu of exposure to nuclear emulsion would be an attractive avenue to pursue. If feasible, this would simplify high resolution autoradiography. However, problems of resolution and sensitivity remain to be solved (Cabello and Wells, 2010). The close contact between tissue section and the recording nuclear emulsion seems prerequisite for the high resolution achieved with the thin thaw-mounted sections. *Electron microscopic autoradiography*, occasionally applied in the past, was expected to provide information relevant to subcellular drug distribution (Ullberg and Appelgren, 1969). Applications have been sparse, and it

appears that no practical elctronmicroscopic method has become available. Depending on the type of compound and purpose of study, low-resolution non-microscopic data as provided by current in vivo imaging methods may suffice for some practical purposes. If used alone, however, false negatives must be considered. Certain questions can be answered only if cellular-subcellular information is available. For obtaining such information and judging from the pictorial evidence, there is currently no feasible, reliable substitute that can replace receptor microscopic autoradiography.

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