Pharmaceutical Research, Vol. 24, No. 5, May 2007 (© 2007)
DOI: 10.1007/s11095-006-9221-0

Commentary

The Origin of Deoxynucleosides in Brain: Implications for the Study of Neurogenesis and Stem Cell Therapy

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Received December 14, 2006; accepted December 15, 2006; published online March 20, 2007

Abstract. Detection of DNA synthesis in brain employing (^{3}H)thymidine ((^{3}H)dT) or bromo deoxyuridine (BrdU) is widely used as a measure of the "birth" of cells in brain development, adult neurogenesis and neuronal stem cell replacement strategies. However, recent studies have raised serious questions about whether this methodology adequately measures the "birth" of cells in brain either quantitatively or in an interpretable way in comparative studies, or in stem cell investigations. To place these questions in perspective, we review deoxynucleoside synthesis and pharmacokinetics focusing on the barriers interfacing the blood-brain (cerebral capillaries) and blood-cerebrospinal fluid (choroid plexus), and the mechanisms, molecular biology and location of the deoxynucleoside transport systems in the central nervous system. Brain interstitial fluid and CSF nucleoside homeostasis depend upon the activity of concentrative nucleoside transporters (CNT) on the 'central side' of the barrier cells and equilibrative nucleoside transporters (ENT) on their 'plasma side.' With this information about nucleoside transporters, blood/CSF concentrations and metabolic pathways, we discuss the assumptions and weaknesses of using (^{3}H)dT or BrdU methodologies alone for studying DNA synthesis in brain in the context of neurogenesis and potential stem cell therapy. We conclude that the use of (^{3}H)dT and/or BrdU methodologies can be useful if their limitations are recognized and they are used in conjunction with independent methods.

KEY WORDS: brain DNA repair; brain ribonucleosides and deoxyribonucleosides; cerebral microvessels; cerebrospinal fluid homeostasis; choroid plexus epithelium; CNT2; CNT3; ENT1; ENT2; nucleoside pharmacokinetics; thymidine kinase; thymidylate synthetase.

INTRODUCTION

Detection of DNA synthesis in brain is widely used as a measure of the replication or "birth" of cells in studies of brain development and adult neurogenesis, and in neuronal stem cell replacement strategies (1–3). In such studies, either (^{3}H)thymidine [(^{3}H)dT] or 5-bromo-2′deoxyuridine (BrdU) (which is covalently incorporated into DNA in place of dT) is injected parenterally (1,2), or one of these deoxynucleosides (dN) is incorporated into stem cell DNA in living cells (3,4). In the latter case, the heavily-labeled stem cells are then injected directly into the brain substance or ventricles (3,4). In both cases, at various times after the injection, the location of the (^{3}H)dT or BrdU is measured in brain cell nuclei by autoradiographic or immunologic methods, respectively (1–5). These methods are widely thought to assess DNA synthesis during the S phase of the cell cycle and thus be a measure of replicative DNA synthesis (1–5). Thus, the "birth" or replication of neuronal, glial and/or endothelial cells during the time of the labeling pulse, a period of up to approximately 2 h (depending on the dose), or after labeled cellular injections, can be detected (1–5).

A large number of valid assumptions, however, is necessary for the (^{3}H)dT or BrdU labeling methodologies to be an accurate measure of replicative DNA synthesis in brain. Unfortunately, some of these assumptions may be or are incorrect. For example, a recent study showed that dividing neuronal precursors in neurospheres, unlike glia, derive their dT from de novo synthesis intracellularly via thymidylate synthetase (TS) rather than from salvage via thymidine kinase (TK) (6). If this happens *in vivo*, parenteral dT might not label such cells. Moreover, another recent study showed that the intraventricular or intraparenchymal injection of heavily-labeled neuronal stem cells (with (^{3}H)dT or BrdU) seemed to divide into easily detectable glial and neuronal precursor daughter cells *in vivo* (3). However, similar results were obtained with heavily labeled dead cells in control experiments (3). These investigations with dead cells starkly point out the dangers of conclusions based on just the use of (^{3}H)dT and BrdU as a measure of transplanted stem cell DNA synthesis (3). Finally, Cameron and McKay showed that in many previous rat studies, the dose of BrdU was too low and could not quantitatively measure adult brain neurogenesis, e.g., in the dentate gyrus (2). Therefore, in comparative analyses with subsaturating doses,

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unless all variables affecting BrdU and deoxynucleotide triphosphate (dNTP) pools in brain cells are the same in both groups, one could easily draw unreliable conclusions.

To place these questions into perspective and to review systematically the assumptions made in the use of (^{3}H)dT and BrdU in neurogenesis and stem cell transplantation studies, we will first cover deoxynucleoside (dN) synthesis and pharmacokinetics including the salvage pathways; the blood-brain (BBB) and blood-cerebrospinal fluid (CSF) barriers to dN transport, especially focusing on the choroid plexus (CP); the early dN transport studies in the central nervous system (CNS) (1975–1990); the more recent clarifying investigations (1991–2006); the mechanisms and molecular biology of transport (especially of dT and BrdU); and the blood levels of dN (and relevant congeners). With this background information, we will discuss the strengths and weaknesses of current methods (including their assumptions) for studying DNA synthesis in brain. The focus will be on the use of dT and BrdU. Finally, we will briefly contrast these labeling methods with other approaches for detecting the birth and division of cells in the CNS. This Commentary emphasizes studies in unanaesthetized animals and man (where possible) and those with sound methodological procedures (e.g., high radiochemical purity of radiolabeled compounds and chromatographic identification of (^{3}H) label in tissues). The main aim of this review is to describe the origin of dNs in the CNS in the context of neurogenesis and potential stem cell therapy.

BIOCHEMISTRY OF dN FORMATION: RELEVANCE TO DNA SYNTHESIS

In general, dN are the moieties transported through various cell membranes, not the phosphorylated forms (with an exception described below). Moreover, in plasma and CSF, the principal forms of ribonucleosides (rN) and dN are nonphosphorylated due to the ubiquitous presence of phosphatases in the extracellular space (7,8).

Although small amounts of dN may be absorbed from the gastrointestinal tract, dN (except dT) are synthesized (mainly within dividing cells) by ribonucleotide reductase (RR), an ubiquitous cytoplasmic enzyme that reduces the four principal nucleoside diphosphates (NDPs; ADP, GDP, CDP and UDP) to the corresponding dNDP (9,10). Other enzymes add or subtract a phosphate to make deoxynucleotide triphosphates (dNTPs) or monophosphates (dNMPs) (9). To synthesize thymidine monophosphate (TMP), cytoplasmic thymidylate synthetase converts dUMP to TMP, using methylenetetrahydrofolate as the methyl donor (9,11). TMP can also be formed by deamination of dCMP by dCMP deaminase intracellularly (9,11).

Beside *de novo* synthesis of dN phosphates, cells, including nondividing ones, have salvage enzymes to phosphorylate dN that enter the cells (generally by facilitated diffusion) (9,12). These are listed in Table I with their abbreviations. TK1 and dCK are highly expressed in dividing cells (like RR and TS), whereas TK2 and dGK are constitutively expressed in mitochondria (which have an intracellular half-life of approximately 30 days) (9,12). Finally, the mitochondrial dNDP exchange carrier that transports dNDP from cytoplasm into mitochondria in exchange for ADP is an exception to the general rule that the nonphosphorylated moiety is the one transported (13). The exchange carrier complements dN transport from cytoplasm into mitochondria by facilitated diffusion (13).

Table I. Deoxynucleoside (dN) Salvage Enzymes and Their Location (9,12,24,31,32)*

Cytoplasm	Mitochondria
Thymidine kinase (TK 1) [dT, dU]	Thymidine kinase (TK 2) [dT, dU, dC]
Deoxycytidine kinase (dCK) [dC, dA, dG]	Deoxyguanosine kinase (dGK) [dG, dA]

*In brackets are the dNs that are phosphorylated.

A question highly relevant to the theme of this Commentary is what happens to cellular DNA synthesis when dT levels are increased outside cells. In dividing cells, extracellular dT (0.1–1.0 mM) stops cell division by raising the intracellular TTP pool, which upsets the delicate balance among the four dNTPs required for DNA synthesis (14). Lower concentrations of extracellular dT do not perturb dividing cells (14). However, lower concentrations (e.g., 10 μM dT) have profound effects on nondividing cells as shown in human thymidine phosphorylase deficiency (14,15). Thymidine phosphorylase irreversibly catabolizes dT to thymine in liver and other organs (14,15). In human homozygous deficiency states, the plasma level of dT increases from a normal of 0.2 to 10 μM (14,15). In resting cells such as muscle and neurons, the consequence is a 4 to 8-fold increase in the dTTP concentration in mitochondria which upsets the delicate dNTP balance (14,15). This leads to a decrease in mitochondrial replication in nondividing cells and an attendant genetic encephalomyopathy (14,15).

BLOOD-BRAIN AND BLOOD-CEREBROSPINAL FLUID BARRIERS

Because the CNS barrier systems help to regulate nucleoside concentrations in the brain interstitial fluid and CSF, it is instructive to delineate structural/functional relationships at these transport interfaces. About a century ago, investigators began to define the BBB and blood-CSF barriers. Over time, they established that the BBB is anatomically due to tight junctions (*zonulae occludentes*) between the brain capillary endothelial cells (16). The blood-CSF barrier is due to tight junctions between the choroid plexus (CP) epithelial cells, and the arachnoid membrane cells (17). The choroid plexus epithelial cells present as a single circumferential layer *versus* multiple stacked layers in the arachnoid membrane (17). The choroid plexus actively secretes the CSF and essential solutes into the ventricles for bulk flow distribution, whereas the arachnoid membrane more passively encloses the subarachnoid CSF compartment that surrounds the brain (17–20).

While the BBB and blood-CSF barriers restrict the diffusion of solutes such as nucleosides between blood and the CNS, the CSF-brain interface does not restrict the diffusion of solutes between the CSF and interstitial space of brain (18). This is due to the more permeable gap junctions at the inner (ependyma) and outer (pia) surfaces of the brain (18–20). Thus, within the CNS, nucleosides can

freely exchange between the brain interstitial fluid and CSF (20).

The surface area of the various transport interfaces is a significant factor for molecular fluxes. The cerebral capillaries (1.0% of brain weight) and the fronded CP tissues in the four ventricles (0.5% of brain weight) have comparable surface areas per unit weight (19). Unlike the brain capillaries, the choroid plexus epithelial cells resemble the proximal renal tubular cells structurally and functionally (21). The reader is referred to detailed up-to-date reviews and diagrams of the anatomical features (19).

In parallel with the anatomical observations, some investigators proposed that the entry (and exit) of molecules into brain and CSF from blood was due to simple diffusion and thus depended on solute charge, molecular size and lipid solubility (21). However, it became clear that a multitude of compounds (including many endogenous substances) do not distribute between brain, CSF and blood according to the postulated rules of simple diffusion based on charge, size and lipid solubility (20,21). Macronutrients, micronutrients including vitamins, "waste products" of metabolism, ions and many exogenous substances seemed to depend on "carriers" to traverse the BBB and blood-CSF barriers (20,21). Over time, from the early studies of glucose and amino acid transport through the BBB by specific carriers, and Welch's documentation of iodide efflux from CSF by a specific, active transport system in CP, several hundred different transport systems have been described or postulated in over 25 general classes (21). Moreover, the specificity of these systems overlaps considerably. Thus a given molecule might be transported via two or more systems.

Another complexity in the CNS is that there are two possible routes of entry into and exit from the CSF and brain, the BBB and the blood-CSF barriers (17,19–21). At first blush, most investigators thought that the BBB would be the quantitatively most important locus of transport especially in adult mammals. But, in 1921, Stern and Gautier presciently proposed that in both the developing fetus and adult, the CSF is "nourishing liquor" for the brain, a hypothesis we now know has merit for several substances (21). Although in most cases transport through the BBB predominates (e.g., influx of glucose and other macronutrients into brain, and efflux of certain xenobiotics and drugs into blood via P-glycoprotein); in other cases, the CP is more important (e.g., influx of reduced folates into CSF, and efflux of penicillin G into blood) (21). As discussed below, both the BBB and CP contain transport systems for dN and rN which, because of their size and water solubility, could not readily pass through the BBB and blood-CSF barriers (except minimally by simple diffusion).

EARLY STUDIES OF dN TRANSPORT AND SYNTHESIS IN CNS (1975–1990)

In this section, we focus on dT for two reasons. First, in mammals, there is no detectable (<0.1 μM) deoxyadenosine (dA) or deoxyguanosine (dG) in plasma and CSF (7,8). Second, although in some species (rabbit and rat) there is readily detectable dC in plasma and CSF, there is minimal dC in mice and humans because of circulating cytidine deaminase in plasma and CSF (7,8).

Focusing on dT which has a plasma concentration of 0.2, 0.6, and 0.8 μM in humans, rabbits, and rats respectively (7,8,22), Cornford and Oldendorf, using a single pass technique, could not detect dT transport beyond simple diffusion through the rat BBB, a result which we confirmed (23,24). However, in substantially longer steady-state intravenous infusion experiments with tracer (^{3}H)dT in conscious adult rabbits, there was substantial entry over 3 h of (^{3}H)dT into CSF and all brain regions analyzed (24). The ratios of (^{3}H)dT in CSF and whole brain to plasma were both 0.7. Thus, in rabbit CSF, with a dT concentration of 0.6 μM, 70% of the dT in CSF entered CSF from plasma during the three-hour infusions (24). In all brain regions tested, there was also a comparable distribution of (^{3}H)dT as well as an equivalent amount of phosphorylated (^{3}H)dT (24). Surprising at the time, raising the concentration of dT in plasma to 15 μM *increased* the penetration of plasma (^{3}H)dT into brain and CSF (24). This was confirmed subsequently by injecting unlabeled thymidine into the ventricles, which also almost doubled the penetration of tracer (^{3}H)dT from plasma into CSF and brain (25). Only when the plasma concentration was raised to 0.5 mM dT was the entry of (^{3}H)dT into brain and CSF from plasma cut by more than half (24).

Even more surprising was the rapidity with which (^{3}H)dT was cleared from the CNS compared to the passive marker (^{14}C)sucrose (24). For example, 2 h after the intraventricular injection of (^{3}H)dT and (^{14}C) into adult rabbits, the ratio of (^{3}H)dT to (^{14}C)sucrose in the tissue compared to that in the injectate was 0.11 and 0.66 in CSF and brain, respectively (24). Only 16% of the injected (^{3}H) was recovered in the CNS *versus* 76% of (^{14}C)sucrose. In the brain about 40% of the (^{3}H) was phosphorylated (24). In some of these studies, highly specific methods were employed to establish that the (^{3}H) in the brain "DNA fractions" was actually (^{3}H)dT covalently incorporated into DNA (1–2%) (26). The rapid clearance (efflux) of (^{3}H)dT from CSF and brain was decreased when carrier dT was added (24). These studies showed that dT was rapidly cleared from the CSF on the one hand; and yet, as noted above, 70% of the dT in CSF came from the blood over 3 h (24).

In brain slices from cortex, cerebellum and brain stem from adult rabbits, (^{3}H)dT was accumulated by an equilibrative (facilitated diffusion) system(s) with subsequent intracellular trapping by phosphorylation by TK (Table I) (24,26). Approximately, 0.1–0.2 μM dT in the medium half-saturated the intracellular phosphorylation and saturable uptake (24,26). The K_M for TK1 is 0.1–0.2 μM (24,26).

In a parallel series of *in vitro* studies of the isolated rabbit CP, we showed that (^{3}H)dT was concentrated by a then novel sodium-dependent active transport system with similar affinity for dN and rN (27–29). This system did not depend on phosphorylation or metabolism of the dN. The K_T (one-half saturation concentration) was 14, 7, and 15 μM for dT, dU, and dC, respectively (27–29). Adenosine (rA), guanosine (rG), dA, and dG had IC_{50}'s (inhibitory concentration 50%) for this system of between 5 and 30 μM (28,29). Moreover, the rapid and saturable release of accumulated (^{3}H)dT, (^{3}H)dU and (^{3}H)dC was partially inhibited by nitrobenzylthioinosine (NBTI) (28,29). Thus, *in vitro*, dN could be transported into CP by a powerful sodium-dependent, concentrative transport system and released in

part by a separate NBTI-sensitive (facilitated diffusion) system (27–29).

Finally, the development of these systems was studied (26). The active nucleoside transport system in rabbit CP was fully active at birth. In developing rabbit, the accumulation and phosphorylation of (^{3}H)dT *in vitro* in brain slices from cortex, cerebellum, and brain stem were similar in developing *versus* mature animals; however, there was an order of magnitude or more greater incorporation of (^{3}H)dT into DNA in both the nuclear and mitochondrial fractions in rabbits less than one week old compared with adult counterparts (26).

A similar series of experiments with brain slices, isolated CP, and *in vivo* in rabbits was performed with (^{3}H)dC and yielded comparable results (29–32). Since dCK (Table I) also phosphorylates dA and dG, these studies provide persuasive evidence for the mechanism by which dA and dG can also be salvaged in brain. (See below.)

One difficulty with these data was that it was not obvious then how to reconcile our data with the Cornford and Oldendorf experiment (23) described above, i.e., that dT does not readily traverse the BBB. So, we wondered in 1980 if the CP possibly was the locus for transferring dT from blood into CSF; then, dT could be taken up by brain (24). Reduced folates are transported into CSF via the choroid plexus in this fashion (21). However, in 1980, we noted that "the amount of (^{3}H) in CSF and brain in the rabbits in group A (tracer infused) is greater than could be accounted for by even the complete extraction and transfer into the CNS of all the (^{3}H)dT in the blood plasma traversing the choroid plexus during the infusion. Thus, other unknown loci in addition to the choroid plexus must be involved in the transfer of (^{3}H)dT from blood into CSF" (24).

In a separate series of experiments to assess the potential synthesis of dN *in situ* in brain, we showed in both rabbits and rats that RR, TS and dihydrofolate reductase (DHFR) were diffusely present and active throughout adult and older brains, although the activities per gram were approximately only 10% those of newborns (10,11). Moreover, we showed that tracer [6-(^{3}H)dU], injected intraventricularly, was rapidly transported out of CSF into blood and brain (33). Some of the dU in brain was converted into (^{3}H)thymidine by TS and subsequently incorporated into DNA as shown by specific methods. These and other experiments unequivocally showed that RR, TS and DHFR were present and active not only in developing but also in adult and old rabbit brains (10,11,33). In fact, 2% of the (^{3}H) in brain cortex (i.e., the tissue above the lateral ventricle) was covalently incorporated into (^{3}H)DNA in adult rabbits after the intraventricular injection of tracer (^{3}H)dU (33).

In summary, these studies taken as a whole showed that the salvage enzymes in Table I were present and active in developing and adult brain for *in situ* salvage. From blood, in the species that have cytidine deaminase, only dT can be salvaged since there is minimal or undetectable dC, dA, or dG in blood and CSF. In developing and adult brain, RR, TS, and DHFR also synthesize dN de novo. However, it was not clear in which cells these enzymes resided, nor was it clear how much dT for DNA synthesis came from blood and how much from *in situ* salvage or *de novo* synthesis in either developing or adult brain.

RECENT STUDIES OF DEOXYNUCLEOSIDE (dN) TRANSPORT IN THE CNS (1991–2006)

Since 1991, a series of sophisticated studies using more sensitive and/or molecular methods have greatly clarified dN transport and metabolism in the CNS. First, the dN transporters at the BBB, CP and CNS cells, including neurons, have been cloned, expressed, defined and localized (34–36). Key dN transporters are shown in Table II (34–36). Concentrative nucleoside transporters (CNT) require energy and one or two sodium ions to function (29,34,36). The equilibrative, low affinity, facilitated diffusion transporters (ENT) do not (26,35).

In BBB analyses, Wu *et al*. (37) used a highly-sensitive *in situ* brain perfusion technique in rats to demonstrate a saturable, low affinity dT carrier. Other investigators observed the transfer of (^{3}H)dT into guinea pig brain (and CSF) by a saturable, partially NBTI-sensitive system with low affinity for dT (38). Moreover, in molecular biological experiments, several investigators found evidence for ENT1, ENT2 and CNT2 in the capillaries of the BBB (22,39–41). The exact location of CNT2 in the cerebral capillaries is uncertain but Redzic *et al*. showed that dA, dG and to a lesser extent rA and rG were either rapidly transported out of the interstitial space of brain into blood or taken up by brain parenchymal cells and presumably salvaged (40). These *in vivo* and subsequent *in vitro* data strongly support (but do not prove) the notion that the CNT2 (Table II) transporter exists on the abluminal (brain) side of the capillary wall (22,41). Thus, *in vivo* there is equilibrative (partially sensitive to NBTI) bidirectional transport of rN and dN by ENT1 and ENT2 between brain and blood at the BBB as well as rapid clearance of extracellular purine rN and dN from brain into blood by CNT2 (Fig. 1) if the rN or dN are not salvaged (24,37–41).

Brain parenchymal cells contain the equilibrative but not the concentrative nucleoside transporters (42). This finding is consistent with previous brain slice data (24,26). Recent histological localization studies have established that both ENT1 and ENT2 are present in neurons; astrocytes contain ENT1, but do not consistently express ENT2 based on histological staining techniques (42). Alanko *et al*. suggested

Table II. Nucleoside (N) Transporters in the CNS (28,29,34–36,41,42)

Concentrative Nucleoside Transporters (CNT)	
Name	Specificity
CNT1 [SLC28a1]	Pyrimidine rN and dN
CNT2 [SLC28a2]	Purine rN and dN
CNT3 [SLC28a3]	Both purine and pyrimidine rN and dN
Equilibrative Nucleoside Transporters (ENT)	
ENT1 (Es)* [SLC29a1]	Broad specificity; low affinity; NBTI sensitive
ENT2 (Ei)** [SLC29a2]	Broad specificity; low affinity; NBTI insensitive

*Es = Equilibrative and sensitive to NBTI.
**Ei = Equilibrative and insensitive to NBTI.

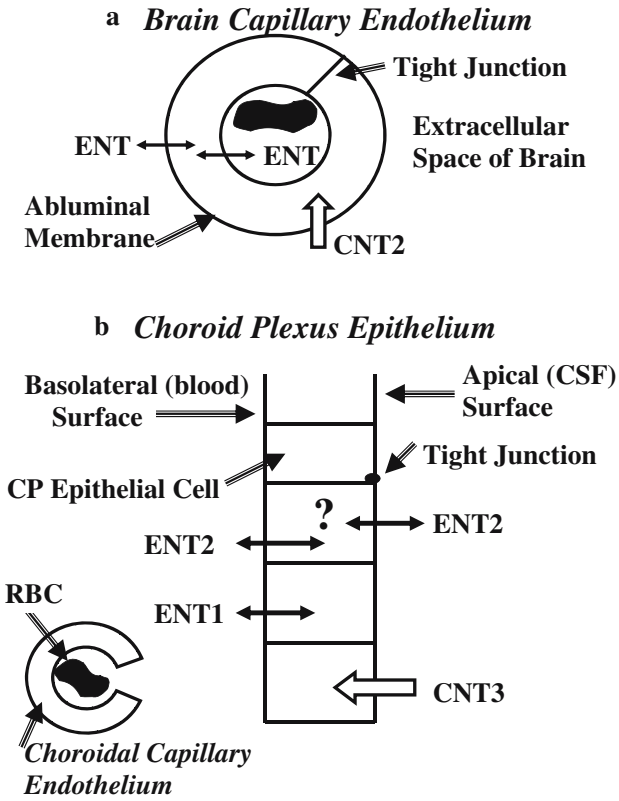

Fig. 1. The postulated carrier mechanisms for transporting nucleosides through the cerebral capillaries and the choroid plexus epithelial cells, both of which are joined by tight junctions, are displayed. In brain capillaries (**a**) there is equilibrative transport (bidirectional transport) of rN and dN on both the blood and abluminal (interstitial space of brain) sides; there is also concentrative transport (*unfilled unidirectional arrow*) of purine rN and dN by CNT2 (Table II) into cerebral capillaries from the abluminal side. In rabbit choroid plexus (**b**) there is concentrative transport of rN and dN by CNT3 (and, in rat, also by CNT2) from the CSF surface, and release of rN and dN from the basolateral side by ENT1. ENT2 is also present in CP but its location needs delineation. The capillaries in the CP unlike brain are not joined by tight junctions (16–21); their permeability (18) allows nucleosides and even macromolecules to readily diffuse between the choroidal interstitial space and capillary blood. RBC, red blood cell.

that ENT1 is the principal cellular plasma membrane transporter in neurons and glia, while ENT2 may subserve intracellular organelles (e.g., mitochondria) which transport dN intramitochondrially (42).

In choroid plexus the function and nature of the active nucleoside transport system have also been clarified. First, Giacomini *et al*. confirmed the existence of a CNT3-like system in rabbit CP and then subsequently described it in rat, primate and human CP (43). They also expressed a CNT3-like transporter from rabbit CP RNA in *Xenopus* oocytes (43). Other investigators cloned, expressed and molecularly characterized the CNT3 system from mice and humans and showed it had the same properties as the rabbit CNT3 system in CP (e.g., required two sodium ions) (36). In rat CP, the CNT3 mRNA seems to be the predominant CNT (mRNA) moiety but CNT2 mRNA is also present (41). CNT2 is also identifiable with polyclonal antibodies in rat CP (41).

Further *in vitro* studies of CP localized ENT1 predominantly to the basolateral surface (blood side) of the CP and CNT to the apical (CSF) side of the CP (Fig. 1) (22). (There may also be ENT2 on both the apical and basal sides of the CP (22).) Therefore, it appears that the rapid clearance of tracer dT, dU and dC from rabbit CSF into blood is due, in large part, to the apically located CNT3 transport system in CP (24,32,33). Unlike the equilibrative systems that are one-half saturated at ~0.2–0.5 mM dT, the CP system is half-saturated at 5 to 20 μM for dN as noted above (28–30). Hence, when the CSF dT concentration is increased above the K_T of the CP system (other nucleosides in CSF like uridine also have affinity for the CNT3 transporter), the CP system becomes saturated and the equilibrative systems at the BBB and blood-CSF barriers predominate (24,25). Hence, the CSF, brain and plasma levels of intravenously infused (^{3}H)dT become comparable (24,25). However, when the plasma levels are raised to ~0.5 mM, there is then partial saturation of the equilibrative systems at both the BBB and CP, and hence the brain and CSF levels of (^{3}H)dT are much lower than that of plasma, 16 and 38%, respectively, after three-hour infusions of tracer (^{3}H)dT (Fig. 1) (24).

Teleologically, it is not clear why the CP should have (a) CNT system(s) facing the CSF. Interestingly, riboflavin transport is like that of dN in that there is a saturable CNS-entry system for riboflavin at the BBB, but also a vigorous clearance system in the CP that removes riboflavin from CSF (21). In the case of dN, it is worth noting that notwithstanding the active, vigorous pumping of dT out of CSF by CP, the

concentration of dT in CSF in both rats and rabbits (at steady state) is the same as that of plasma (7). In the case of both riboflavin and dT, there is very rapid turnover of these molecules in both the CSF and interstitial space of brain for inexplicable reasons (21,24).

BLOOD LEVELS OF dN AND rN

In humans and rabbits, the plasma concentration of dT is 0.2 and 0.6 μM respectively (7,8,44). However, some investigators find human plasma dT <0.05 μM. The reasons for these discrepancies are unclear but in one case, the investigators did not neutralize perchloric acid precipitates of plasma and injected the supernatant directly into the HPLC column (15). This procedure may spread out the dT peak and minimize the detection of dT at low concentrations. The plasma concentration of dT is kept remarkably constant even in the face of 4 days of only water (rabbits) (45) or 5 days of 400 carbohydrate calories daily (humans) (46). The only measurable nucleoside to change in both rabbits and humans was plasma uridine which decreased by 30–40% in both species (45,46). Moreover, in rabbit CSF, there were no changes in rN or dN after 4 days of water intake (45). Thus, the body has complex but effective short term homeostatic mechanisms to keep stable plasma and CSF dN and rN concentrations (except plasma uridine) normally. Even after constant intravenous infusions into conscious rabbits of 500 mg thymidine/kg over 5 h, the concentrations of measurable rN and dN in plasma and CSF (except dU, dT and thymine which were greatly increased) were changed remarkably little, thus attesting to the efficacy of the endogenous nucleoside homeostatic systems (45). However, the effects of stress, chronic weight loss, steroid injections, and other manipulations on dT (or BrdU) pharmacokinetics in blood, CSF and brain all await elucidation.

BrdU PHARMACOKINETICS AND INCORPORATION INTO DNA

Although BrdU can be covalently incorporated into DNA in place of dT, little is known of BrdU pharmacokinetics. In humans and primates, the plasma half-life after intravenous injection is ~10 min (2). It is assumed that BrdU behaves like dT (2). Evidently the only transport data on this point is that BrdU has an inhibitory affinity (IC_{50}) for the active rabbit CP transport system (CNT3) of 9 μM; in comparison, that for dT is 14 μM as noted above (2,29). Whether BrdU is actually transported by the ENT or CNT systems is unknown but seems likely. As noted above, BrdU and (^{3}H)dT, incorporated covalently into DNA, can exit from heavily-labeled dead neuronal stem cells *in vivo* (3). The BrdU or dT can then be accumulated by surrounding neuronal/glial cells via salvage pathways (Table I) to label the dividing cells detectably (3).

IMPLICATIONS OF USING dT OR BrdU TO MEASURE DNA SYNTHESIS IN BRAIN

In adult mammals, neuronal precursor cell division (outside the subventricular zone/ olfactory system) unequivocally occurs only in the dentate gyrus (1,2,5). Intense efforts have been made to study dividing neuronal cell precursors and their stability in brain, especially in the dentate gyrus *in vivo* in both developing and adult animals. Changes in dentate gyrus neurogenesis after manipulations of the environment or after drug administration have been repeatedly reported (1,2,5). These studies principally employed BrdU (and to a lesser extent dT) labeling techniques (1,2,5). There are, however, many important unknown pharmacokinetic and potential complicating factors with these techniques (Table III). Until recently in adult rats, the optimal dose of BrdU for labeling dividing cells (e.g., in the dentate gyrus) was not carefully assessed (2). Moreover, different species of rodents show large differences in adult neurogenesis with the same dose of BrdU (47). The reasons for these differences are not clear; possibilities include pharmacokinetic or other genetic aspects of rat and mouse biology. Consequently, many studies, which used too low doses, almost certainly underestimated adult neurogenesis (1,2,5). Furthermore, comparative studies that employed too low doses assume that both the pharmacokinetic and potential confounding factors (Table III) are the same in both groups. With subsaturating amounts of BrdU, this is a very unlikely assumption in our view, e.g., in rats in enriched *versus* caged environments, or rats on profoundly different diets with different weights. Even with saturating doses there may be differences in other variables, e.g., blood flow.

In the last decade, many studies of enriched environments, stress, exercise, learning and corticosteroids, or drugs to treat depression and mania, have suggested that these interventions alter adult neurogenesis in the dentate gyrus (assessed by BrdU labeling), although many of the studies are contradictory (1,48). For example, in some investigations, enriched environments increase whereas in other studies, they decrease dentate gyrus neurogenesis measured by BrdU methodology (49). Corticosteroids supposedly decrease dentate gyrus neurogenesis but exercise, which increases steroid levels substantially, increased neurogenesis (1). Clearly there are too many variables to trust BrdU labeling alone to draw firm conclusions. These questions about the effects of various interventions on dentate gyrus neurogenesis await definitive clarification. We suggest that other methods should be employed to corroborate the conclusions about the effects of stress, enriched environments, steroids, antidepressants, lithium and exercise on adult neurogenesis as assessed by BrdU. Such methods include detection of Ki-67, a nuclear antigen, which appears during cell division, and cytoplasmic RR which increases by orders of magnitude during cell division (50,51), or the use of reporter genes in stem cell studies (3,4).

The notion that BrdU is stable in brain cells is also only an assumption (Table III). In some cells, BrdU is removed after covalent incorporation (52). Moreover, it is clear that previously-labeled dead cells can "pass on" their labels to viable cells which then become labeled (3). Thus, weeks or months after pulse labeling, the finding of labeled cells does not prove the cell was originally labeled during the pulse, and that the BrdU label was stable.

The potential for some dividing neuronal precursors to favor preferentially de novo synthesis of dT by TS and not salvage (via TK 1) is problematic. In neurosphere experiments, folate (but not dA+dT+dG+dC) in the medium was

Table III. Various Factors That Affect the Use of BrdU to Label the Brain DNA

	Pharmacokinetic Factors
1)	Plasma levels of dT (and competing rN) and BrdU
2)	Influx transport at BBB
3)	Efflux transport at CP
4)	Distribution of BrdU in brain
	a) Due to blood flow/local permeability/transport
	b) With changes in blood flow/transport due to perturbations
5)	Transport into and salvage by brain cells
6)	Triphosphorylation of BrdU
7)	Size of TTP and BrdUTP pools in neurons and other brain cells
	Potential Complicating Factors
1)	Preferential *de novo* synthesis (TS) with lack of BrdU salvage/incorporation into DNA in some cells
2)	Stability of BrdU incorporated into DNA (e.g., uracil glycosylase removal)
3)	Mutagenic/toxic potential of BrdU in DNA
4)	BrdU for mitochondrial *versus* nuclear DNA synthesis
5)	Affinity of BrdUTP *versus* TTP for polymerases
6)	Possibility that high plasma BrdU levels perturb DNA synthesis in brain cells
7)	Release of BrdU from covalently labeled DNA in cells, and reuptake by and labeling of other cells
8)	Criteria for deciding when and what cells are labeled

able to support replication of neuronal precursors (6). This was not due to homocysteine toxicity. These experiments provide strong although not conclusive evidence for the decisive role of TS in neuronal precursor cell division (6,11). If *de novo* synthesis predominates in some brain cells *in vivo*, then labeling with tracer dT or BrdU may be "blind" to these cells. *In vivo* work is required to establish this possibility and, if true, its implications. Pretreatment with 5-fluorouracil, for example, to block TS activity (as done in tissue culture studies of DNA synthesis with BrdU) might foster dT or BrdU uptake in brain cells where TS may normally predominate (52). Clearly there are significant quantities of TS and DHFR in the brain at all ages (11), and a very powerful folate homeostatic system, predominately in the CP, to ensure stable quantities of reduced folate for the brain (21). Moreover, the enzymes RR, TS and DHFR and the reduced folate transport system play an important role, since blockade or dysfunction of these systems due to drugs or genetic abnormalities produce substantial CNS toxicity (21). However, it remains possible that in adult mammals, outside of the dentate gyrus and olfactory system, the principal normal role of RR and TS may be to make dN for incorporation into DNA for nuclear repair, mitochondrial replication and the occasional glial or endothelial cell division; not for de novo dN synthesis in neuronal precursor cells for replication due to the insufficient activity of TK 1.

The issue of the distribution of labeled dT and BrdU in brain after parenteral injection is also not clear. This needs resolution in order to interpret reliably certain neurogenetic analyses. Is there preferential distribution of dT or BrdU to specific brain areas or cells, and, thus, the appearance of more labeling? Does dT or BrdU labeling preferentially occur in cells near vessels (53)? Moreover, changes in local blood flow in certain areas (due to shunting of blood to a greater number of open capillaries in those regions), e.g., during stress or learning, could have profound effects on dT or BrdU labeling (Table III).

In the dead stem cell study referred to above (3), the importance and efficiency of the salvage pathways for dT and BrdU (Table I) are exemplified in that the heavily labeled dead stem cells, after intraparenchymal or intraventricular injection, passed their label to glial and neuronal precursors. Almost certainly, these dead cells were degraded, presumably by enzymes (including DNAase, phosphodiesterases and phosphatases) that released (^{3}H)dT or BrdU for salvage by nearby living cells, and incorporation into DNA (3,24–26). Other methods (e.g., the use of reporter genes in stem cells) can avoid misidentification of stem cell proliferation by not relying too heavily on putative (^{3}H)dT or BrdU incorporation into DNA (3,4). Moreover, in the adult dentate gyrus, many new-born neurons normally die soon after birth, a situation exacerbated by experimentally-induced seizures (1). What happens to the BrdU from the neuronal nuclei of previously heavily labeled dying cells in experimental studies of the dentate gyrus? This question needs to be addressed.

One additional potentially complicating factor in all studies of neurogenesis is the difficulty in deciding in which cells the label exists, and the nature of the cell, whether neuronal, glial, microglial, or endothelial cell. This issue is beyond the scope of this paper, but has been reviewed elsewhere (1,5).

CONCLUSION

Strides have been made in understanding the origin of the natural dN in brain via *de novo* synthesis *in situ*, dN reutilization by salvage *in situ*, or transport from the blood. At the BBB, blood-CSF barrier and in brain parenchymal cells, both the concentrative and equilibrative nucleoside transporters have been cloned and identified. Several unexpected discoveries have emerged (e.g., the active nucleoside transport system (CNT) on the apical side of the CP that rapidly removes dN and rN from CSF). Therefore, a role for choroidal CNT in the CSF homeostasis of nucleosides is surmised. However, there are still many unanswered questions with dT (e.g., the regional distribution of dT in brain after parenteral injection, the effect of blood flow, and the relative importance of TS *versus* TK in individual dividing

cells). This information will further enrich adult neurogenesis studies. Much less is known about the use of BrdU to label brain cells (Table III). However, methods are now available to investigate and resolve the unanswered questions. Such studies are essential for developing successful paradigms of adult neurogenesis and potential stem cell therapy.

ACKNOWLEDGEMENTS

The authors thank Michiko Spector for her aid in the preparation of the manuscript and Julie Johanson for assistance with graphics.

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