Acetylcholine Release and Choline Availability in Rat Hippocampus: Effects of Exogenous Choline and Nicotinamide

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ABSTRACT

The influence of choline availability on acetylcholine (ACh) release in the hippocampus of the awake rat was investigated using the microdialysis procedure. Three treatments enhancing choline availability for basal and atropine-evoked ACh release were evaluated: acute administration of choline chloride (20 mg/kg i.p.); pretreatment of animals with nicotinamide (10 mmol/kg s.c.) 2 hr before atropine injection and dietary choline supplementation (5-fold increase of choline intake for 15–18 days). Although acute choline administration led to a short-lasting (15 min) increase of basal choline efflux by 25% and nicotinamide caused a long-lasting (5 hr) increase by 105%, neither one affected basal ACh release. However, basal release of choline (1.38 pmol/min) and of ACh (114 fmol/min) in the hippocampus was slightly increased in choline-supplemented animals (choline: 1.92 pmol/min; ACh: 140 fmol/min). In untreated animals, atropine administration caused a 3-fold increase of ACh efflux that lasted approximately 2.5 hr. All treatments, acute or chronic choline and nicotinamide, led to significant increases of the maximum and duration of atropine-evoked ACh release. Total atropine-evoked ACh efflux (area under the curve) was increased 2–3-fold, with the largest effect evoked by the combination of nicotinamide and choline. The results clearly demonstrate that, under stimulated conditions, hippocampal ACh release could be facilitated when the availability of choline for ACh synthesis was enhanced by dietary or pharmacological means. Under certain conditions, significant effects of increased choline availability on ACh release can be revealed in the absence of an overall increase of extracellular choline.

The dependence of learning and memory processes on an intact hippocampal and cortical cholinergic innervation, and the central cholinergic dysfunction associated with senile dementia led to the "cholinergic hypothesis of geriatric memory dysfunctions" (Bartus et al., 1982; Perry, 1986). According to the concept of a "precursor control of neurotransmitter release" (Wurtman et al., 1981), dietary supplements of choline and lipid-bound forms of choline were suggested as a possible therapeutic approach to improve symptoms of cholinergic deficiency, and numerous experimental studies were performed investigating the effect of choline administration on the content, synthesis and release of ACh in the brain (for review see Wecker, 1990). Several studies suggested that the synthesis and release of ACh is influenced by choline administration in the striatum where the cholinergic innervation density and the ACh turnover rate is high. Thus, oral and systemic choline administration increased striatal ACh levels (Haubrich et al., 1975; Hirsch and Wurtman, 1979). In striatal slices, both basal and stimulated ACh release was elevated in the presence of >10 μM choline in the superfusion fluid (Maire and Wurtman, 1985; Ulus et al., 1989). In vivo, in awake animals, choline enhanced basal and/or stimulated ACh efflux from the striatum and N. accumbens (Westerink and de Boer, 1990; Farber et al., 1993; Rada et al., 1994; Buyukusal et al., 1995). Striatal ACh release was also increased after i.c.v. administration of choline while hemicholinium-3, an inhibitor of cellular choline uptake, decreased ACh release (Koshimura et al., 1990). Finally, in the reverse case, the decrease of brain extracellular choline observed after i.v. treatment with choline oxidase also led to a decrease of striatal extracellular ACh (Ikarashi et al., 1994).

However, in the regions that are more important for cognitive functions, i.e., hippocampus and cortex, the situation is more complicated. In contrast to the data observed in the striatum, choline usually had no effect on ACh content or release in the nonstimulated hippocampus (Wecker, 1990). However, when ACh release was stimulated by the administration of atropine, pentylenetetrazol or fluphenazine, the resulting reduction of ACh content in both striatum and

ABBREVIATIONS: ACh, acetylcholine; AUC, area under the concentration vs. time curve; CNS, central nervous system; CSF, cerebrospinal fluid; HACU, high affinity choline uptake; HPLC, high-pressure liquid chromatography.
hippocampus was prevented by prior administration of choline (Trommer et al., 1982) or phosphatidylcholine (Jope, 1982). These findings suggested that an increased choline availability may increase the synthesis and release of ACh in the hippocampus under conditions of increased neuronal firing. Interestingly, the effects of choline administration were measurable long after free choline levels had returned to normal, indicating that supplemental choline may be stored in a precursor pool that subsequently can be mobilized to support ACh synthesis (Schmidt and Wecker, 1981; Wecker et al., 1989). It also deserves mention that only hippocampal, but not striatal slices were able to store and mobilize surplus choline for ACh synthesis (Wecker et al., 1989). In in vitro experiments, exposure of slices to choline concentrations of >10 μM was necessary for facilitated ACh synthesis at later time points (Wecker, 1991).

The current state of knowledge seems to suggest that an increase of extracellular choline in the hippocampus may well improve ACh synthesis and increase stimulated ACh release, provided that extracellular choline concentrations of at least 15 to 20 μM are reached. Our recent work, however, indicated that increases of brain extracellular choline after acute choline administration are limited in extent. In untreated animals, a net uptake of choline into the brain is observed at plasma choline levels of more than 14 μM (Köppen et al., 1990). However, even a massive net uptake of choline from arterial blood caused only small increases of free choline in brain tissue and extracellular space (Klein et al., 1992; Köppen et al., 1993). The mechanisms responsible for the rapid removal of brain extracellular choline include rapid cellular uptake and phosphorylation of choline and rapid removal of surplus choline from the brain into the circulation via the cerebrospinal fluid (Klein et al., 1992; Löffelholz et al., 1993). To affect the homeostatic mechanisms that regulate the concentration of brain extracellular choline we have previously used nicotinamide as a model drug. Nicotinamide, a vitamin of the B group, had been reported to enhance choline availability and to support ACh synthesis (Schmidt and Wecker, 1981; Wecker et al., 1990, 1992; Löffelholz et al., 1993). In the acute experiments (figs. 1–3), the animals received the following injections: nicotinamide and mannitol (10 mmol/kg) were dissolved in water and administered s.c., choline chloride (20 mg/kg) and atropine (5 mg/kg) were dissolved in saline and administered i.p. When the effects of choline on stimulated ACh release were tested, choline was administered together with atropine. When the effects of nicotinamide on stimulated ACh release were tested, nicotinamide was administered 2 hr before atropine. To evaluate the combination of nicotinamide and choline on stimulated ACh release, the rats first received nicotinamide and then, 2 hr later, a combination of atropine and choline.

In an additional set of experiments (fig. 4), we tested the effect of dietary choline supplementation. For this purpose, choline chloride (10 g/liter) was added to the drinking water for 15 to 18 days. This mode of application caused about a 5-fold increase in the nutritional choline intake compared to animals on standard diet (cf., Bartus et al., 1980; Klein et al., 1991). Choline supplementation was terminated 12 to 16 hr before the microdialysis experiment to ensure that chronic rather than acute choline effects were evaluated.

Presentation of results. All results are presented in % ± S.E. of number (N) of experiments. Statistical analysis was performed by one-way ANOVA (fig. 5) or by two-way analysis of variance for repeated measurements (figs. 1, 3 and 4).

Results

Effects of atropine administration. In the presence of 10 μM neostigmine in the perfusion solution, the basal rates of eflux from the hippocampus of untreated rats were 114 ± 7 fmol/min ACh and 1.38 ± 0.09 pmol/min choline (N = 47). The i.p. injection of atropine (5 mg/kg) increased ACh eflux (fig. 1). The maximum increase of ACh eflux (365 ± 61%) was observed 30 min after atropine administration, but the atropine-induced ACh eflux was still enhanced after 3 hr (167 ± 13%). Atropine did not affect the eflux of choline in any of the experiments (cf., fig. 2).

Effects of acute choline administration. The acute administration of choline chloride (20 mg/kg i.p.) caused a small and short-lasting increase of basal choline release by
variance for repeated measurements. Each value represents the mean ± S.E. of three to five experiments.

Fig. 1. Efflux of acetylcholine (ACh) into the hippocampal dialysate: Effect of acute choline administration. Rats received i.p. injections of choline chloride (20 mg/kg), atropine (5 mg/kg) or the combination of atropine + choline at time zero. Data are presented as percent of the basal efflux (114 ± 7 fmol/min, N = 47). Each value represents the mean ± S.E. of 5 (choline) or 10 (atropine and atropine + choline) experiments. Statistical comparison between the responses of atropine vs. atropine + choline: F1,179 = 44.9; P < .001 by two-way analysis of variance for repeated measurements. 6.83; P < .010 by two-way analysis of variance; in particular, the time needed to attain maximum ACh release appeared to be shortened by the coadministration of choline (fig. 3).

Effects of dietary choline supplementation. Dietary choline was supplemented by adding 10 g/liter of choline chloride to the drinking water, but choline-containing water was removed at least 12 hr before the experiment was conducted. In a previous study (Klein et al., 1991), we reported that, under these conditions, the plasma choline levels remained slightly elevated (1.5-fold higher than rats on control diets) whereas the CSF choline levels were doubled (15.3 μM in choline-supplemented rats vs. 7.7 μM in control rats). In our experiments, the diet-induced increase of brain extracellular choline was reflected in an increase of basal hippocampal choline efflux by 38% to 1.92 ± 0.24 pmol/min (N = 7; P < .05). This efflux remained at a steady level during the time of measurement (not illustrated).

Fig. 2. Efflux of choline into the hippocampal dialysate. Rats received nicotinamide (10 mmol/kg) or mannitol (10 mmol/kg) at time zero. Two hours later, some animals (closed symbols) received atropine (5 mg/kg, i.p.) as indicated by the arrows. Data are presented as percent of basal efflux (1.38 ± 0.08 pmol/min, N = 47). Each value represents the mean ± S.E. of three to five experiments.

Fig. 3. Efflux of acetylcholine (ACh) into the hippocampal dialysate: effect of nicotinamide. In control experiments, rats received an injection of nicotinamide (10 mmol/kg s.c.) at time zero (“nicotinamide”). For the measurement of stimulated ACh efflux, rats received nicotinamide (10 mmol/kg s.c.) at -120 min and, 2 hr later at time zero, an i.p. injection of 5 mg/kg atropine (“nicotinamide + atropine”) or 5 mg/kg atropine + 20 mg/kg choline chloride (“nicotinamide + atropine + choline”). Data are presented as percent of the basal efflux (114 ± 7 fmol/min, N = 47). Each value represents the mean ± S.E. of eight experiments. Statistical comparison between the responses of “nicotinamide + atropine” vs. “nicotinamide + atropine + choline”: F1,143 = 6.83; P = .010 by two-way analysis of variance for repeated measurements.
In choline-supplemented animals, the basal ACh release amounted to $140 \pm 16$ fmol/min (100% in fig. 4) which is somewhat higher than that of the control rats ($114 \pm 7$ fmol/min, $N = 47$); however, the difference is not significant ($P > .1$). Atropine induced an elevation of ACh release (maximum $563 \pm 32\%$ after 30 min) which was of longer duration and considerably higher than that observed in animals kept on the standard diet ($365\%;$ fig. 4). This atropine effect was significantly stronger in choline-supplemented rats than in controls ($P > .001$). The administration of saline led to a short-lasting increase of ACh by $57 \pm 26\%$ 15 min past injection (fig. 4).

**Comparative evaluation.** The effects of acute choline administration, nicotinamide administration and dietary choline supplementation on the stimulated hippocampal ACh release were further analyzed by the calculation of the amount of ACh released and recovered in the microdialysis probe in the 3 hr after the injection of atropine. For this purpose, the AUC was calculated for each individual experiment by calculating the AUC of stimulated ACh release and subtracting the AUC of basal release. The results were expressed in arbitrary units, and the AUC values of individual experiments were pooled for each treatment group. The results of this comparative evaluation (expressed as mean $\pm$ S.E.) are illustrated in figure 5. It is evident that all treatments aimed at increasing brain extracellular choline led to a significant increase of stimulated ACh release. Although the differences between the treatment groups were not significant, the pretreatment of animals with nicotinamide seems to be a particularly efficient way to enhance stimulated ACh release in the hippocampus. In the presence of nicotinamide, coadministration of choline led to a further, slight increase of ACh release ($P > .1$ by $t$ test).

**Discussion**

**Basal release of acetylcholine.** Numerous experimental studies have tested the possible effect of choline administration on the synthesis and release of ACh in the brain. Although variable results were obtained by different investigators (Wecker, 1990), an influence of the precursor choline on basal ACh release could be convincingly established only for striatal cholinergic interneurons (see above). Our study shows that even the long-lasting increase of extracellular choline observed after nicotinamide administration (fig. 2) did not cause a parallel increase of basal ACh release in the hippocampus (fig. 3). This means that an increase of extracellular choline by a factor of 2 does not affect basal ACh release in the hippocampus of awake animals, and this conclusion is in agreement with previous studies on ACh tissue levels in this brain region (Wecker, 1990). It is worth noting, however, that a possible effect of choline on ACh release may have been masked by the presence of neostigmine, as shown previously in the striatum (Marshall and Wurtman, 1993). Moreover, an increase of basal ACh release due to choline administration was detected by microdialysis in the striatum in some (Farber et al., 1993; Buyukusal et al., 1995) but not in all studies (Westerink and de Boer, 1990). In our experiments, the acute administration of saline, choline or nicotinamide led to a short-lasting (15 min) increase of ACh release (by 25–50%) that we attribute to an unspecific arousal reaction caused by the injections. Increases of hippocampal ACh release by arousal have been reported previously (Day et al., 1990; Nilsson et al., 1990).

**Stimulated release of ACh.** In contrast to the situation in the striatum (see above), it remains questionable whether an increase of choline availability could facilitate the stimulated release of ACh from other areas of the brain, such as the long projection fibers of the septohippocampal pathway which are intimately involved in cognitive processes. Especially the nature of the term “choline availability,” and the relationship of choline availability to extracellular choline concentration awaits further characterization. Therefore, in our study, three different approaches to enhance choline availability have been comparatively analyzed for their effect on stimulated ACh release. For this purpose, ACh release was stimulated by i.p. injection of 5 mg/kg atropine that caused a long-lasting increase of hippocampal ACh release by...
blocking muscarinic autoreceptors. The effect of atropine was further enhanced in the presence of the cholinesterase inhibitor, neostigmine, in the perfusion fluid (Messamore et al., 1993; Liu and Kato, 1994). In untreated animals, atropine led to a 3-fold increase of hippocampal ACh release that lasted for approximately 150 min. Irrespective of the mode of treatment, acute administration of choline or nicotinamide or high-choline-diet, all treatments significantly enhanced the stimulated release of atropine (figs. 1, 3, and 4). In fact, the maximum release of ACh was remarkably similar in all treatment groups (570, 524, 557 and 563% of basal values after administration of choline, nicotinamide, a combination of nicotinamide and choline, and dietary choline supplementation, respectively). In addition, the total extent of evoked ACh release (AUC; fig. 5) did not differ between the treatment groups. Thus, the synthesis and release of hippocampal ACh was facilitated by increases of choline availability, irrespective of the mode of treatment and the increase of the extracellular choline concentration (see below).

**Effects of acute choline administration.** A prominent role of the extracellular choline concentration for ACh synthesis has been inferred from studies with hemicholinium-3, an inhibitor of the high-affinity choline uptake of cholinergic nerve terminals (Tucek, 1988; Koshimura et al., 1990). In the case of acute choline administration, we have reported here and in detail in an earlier investigation (Köppen et al., 1993) that 20 mg/kg choline chloride led to a minor (25%) and short-lasting increase of hippocampal extracellular choline. However, its effect on the atropine-evoked ACh release lasts for more than two hours (fig. 1). At this time point, exogenous choline is mostly present in the form of phosphocholine although metabolism into phosphatidylcholine has only occurred to a small degree (Klein et al., 1992). Thus, it may be hypothesized that under conditions of increased ACh synthesis, choline may be recovered from an intermediate precursor pool (possibly phosphocholine) to preferentially support ACh synthesis; this interpretation is in agreement with previous studies on the temporal dependence of the effects of acute choline administration (Schmidt and Wecker, 1981; Wecker et al., 1989; Jackson et al., 1995). The formation of phosphocholine in synaptosomes, and the presence of choline kinase in this preparation, have been reported (Abdel-Latif and Smith, 1972; Reinhardt and Wecker, 1983). However, it is currently unknown whether phosphocholine can be converted back to choline within the synaptic nerve ending.

**Effects of dietary choline supplementation.** A different mechanism of action is probably involved in the effect of dietary choline supplementation, because the surplus choline that is responsible for the increased availability of choline used under this condition likely stems from lipid-bound choline. Previous experiments showed that labeled choline taken up by the brain is slowly (within 24–72 hr) incorporated into phospholipids (mainly phosphatidylcholine) (Jope and Jenden, 1979; Klein et al., 1992), from where it is released in a delayed fashion. Uptake and release of choline in the brain are in a dynamic equilibrium, and the release of surplus choline from the brain is reflected in a negative arteriovenous difference of brain choline (Klein et al., 1990, 1991). Wecker (1985) reported increased concentrations of phosphatidylcholine and lipid-bound phosphorus in animals on a choline-enriched diet. We have previously reported (Klein et al., 1991) that dietary choline supplementation (identical to the regimen applied in our study) led to an increase of CSF choline by a factor of 2 and an increase of choline release into the venous effluent of the brain. In our experiments, a significant, 38% increase of extracellular hippocampal choline was observed in choline-supplemented animals. It appears doubtful that this limited increase of extracellular choline was sufficient to facilitate ACh synthesis in cholinergic terminals to the observed extent (fig. 4). Rather, it may be hypothesized that the increase of cholinergic firing somehow led to a local mobilization of choline from lipid stores that may be restricted to the synaptic region and is not detectable by the microdialysis procedure. However, evidence for the latter hypothesis is scarce. There have been reports that freshly synthesized ACh contains choline moieties that have been released from phosphatidylcholine by the action of phospholipase D (Hattori and Kanfer, 1985; Lee et al., 1993), but there is no evidence that this process is fast enough to play a significant role in the rapid modulation of ACh release during neuronal firing. A previous hypothesis that synaptic ACh may activate phospholipase D and mobilize choline (Löffelholz, 1987) has been difficult to prove experimentally (Klein et al., 1996).

**Effects of nicotinamide.** Although intermediate stores of choline have to be discussed to explain the effects of acute or chronic choline on ACh release, the effect of nicotinamide can be explained in a straightforward manner by the long-lasting enhancement of the extracellular choline concentration after nicotinamide administration. Nicotinamide injection led to an elevation of extracellular choline to more than 200% of basal values which lasted for more than 3 hr (fig. 2), in agreement with our previous results (Köppen et al., 1993). After atropine stimulation, nicotinamide-treated animals released an amount of ACh that was approximately two times higher than that measured in control experiments (figs. 3 and 5). Thus, in the case of nicotinamide, a doubling of extracellular choline was directly reflected in a parallel increase of ACh output. Interestingly, the combined treatment of nicotinamide and choline led to a further, statistically significant increase of ACh release (fig. 3). However, this further increase was of a small extent, compared to the strong synergistic effect of the combination of nicotinamide and choline on the extracellular choline concentration observed in our previous study (Köppen et al., 1993). This result seems to suggest that an increase of extracellular choline by 2-fold is sufficient for a maximal, choline-induced facilitation of ACh release, and further increases of choline cannot be expected to yield major effects.

What is the mechanism by which nicotinamide increases extracellular choline? Jenden et al. (1990, 1991) mused that N-methyl nicotinamide may be the responsible metabolite; N-methyl nicotinamide is known to inhibit the choline transport and reduce choline clearance out of the brain (Lamann and Schanker, 1980). Vargas and Jenden (1996) recently reported that an enzyme which methylates nicotinamide is present in brain cytosol. Preliminary results from our laboratory, however, indicate that nicotinamide may also actively mobilize choline from choline-containing phospholipids (Erb, C., unpublished experiments).

**Role of high-affinity choline uptake.** From our in vivo results it is concluded that the improved availability of choline in the brain extracellular fluid increases ACh synthesis and release from hippocampal cholinergic nerve endings un-
der stimulated conditions. This result is in agreement with a recent study (Jackson et al., 1995) in which significant effects were seen with large doses of choline (60–120 mg/kg i.p.) given 1 hr before scopolamine. The finding that an increase of brain extracellular choline can lead to enhanced ACh synthesis is at variance with a previous hypothesis claiming that the HACU located at cholinergic nerve terminals is the rate-limiting step for ACh synthesis (for review, see Tucek, 1988).

As the extracellular choline concentration in the brain (3–6 μM) is larger than the $K_c$ of the HACU (1–2 μM) it was assumed that, under physiological conditions, this carrier would work under saturating conditions making an influence of supplemental choline unlikely. However, it is possible that the extracellular choline concentration falls below the $K_c$ of the HACU under conditions of increased neuronal demand, in which case supplemental choline would increase choline transport through the HACU (Lindmar et al., 1980). Unfortunately, there is no method available at present to measure choline levels in the immediate vicinity of cholinergic terminals. As an alternative explanation, one might assume that surplus choline can be taken up into cholinergic neurons via the low-affinity choline carrier. It has been shown that extracellular choline can be used for ACh synthesis even when the high-affinity carrier is completely blocked (Suzuki et al., 1989), and the low-affinity carrier is present on purified cholinergic synaptosomes (Richardson, 1986).

**Therapeutic implications.** Therapeutic trials using choline or lecithin monotherapy for central cholinergic dysfunctions have been largely unsuccessful (for review see Kumar and Calache, 1991). However, experimental studies indicate that an increased rate of ACh synthesis occurs in cholinergic neurons after partial lesions of the septohippocampal pathway (Laphak et al., 1991; Leanza et al., 1993), and our study shows that, under conditions of rapid ACh turnover, hippocampal synthesis and release of ACh can be facilitated by increasing choline availability. Thus, choline availability may be a potential target as an (adjuvant) therapy for central cholinergic dysfunctions, if the pharmacologically induced elevation of the hippocampal ACh release is assumed to reflect pathophysiological conditions. Our results demonstrate for the first time that a dietary administration of supplemental choline can have similar effects on ACh release as acute administrations of high doses, and that a pharmacological manipulation of brain extracellular choline is equally or more efficient than choline in facilitating hippocampal ACh release. Importantly, the availability of choline for ACh synthesis can be increased in the absence of overall increases of the extracellular brain choline concentration, and effective pharmacological interventions may therefore be aimed at increasing the levels of free as well as bound choline.

**References**


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