with age from 4 to 16 weeks. The mice used in the present experiment were about 30 weeks of age. Their sleeping times were increased, apparently as a function of age, from 90 minutes at 16 weeks to 130 minutes at 30 weeks in the BALB/cCrgl mice, and from 25 minutes to 45 minutes in the C57BL/Crgl mice.

The results of the blood-alcohol analysis are presented in Table 2. The difference between the blood-alcohol levels in the two strains at waking, when the righting response is regained, is significant by the Mann-Whitney U test ($p < .001$). The values obtained 3 and 4 hours after injection are not statistically different for the two strains (by the *t*-test), indicating that the rates of alcohol metabolism are not significantly different during this period. The level of brain-alcohol is the same in both strains at 40, 100, and 140 minutes (Table 3). However, the difference in brain-alcohol levels of C57BL/Crgl mice at 40 minutes after injection and BALB/cCrgl mice at 140 minutes after injection is highly significant ($p < .001$) when each regains its righting response.

The results of this study confirm the findings of McClearn (*4*) and Kakihana *et al.* (*8*) that genotype and age of the animals are important determiners of response to ethanol in mice. The finding of the present study that the C57BL/Crgl and BALB/cCrgl strains awaken at different blood-alcohol levels suggests that brain sensitivity to ethanol is different in the two strains. This hypothesis is further supported by the result that C57BL/Crgl mice awoke at significantly higher brain-alcohol levels than did BALB/cCrgl mice. The hypothesis that differences in brain sensitivity to ethanol underlie the difference in sleeping time is thus strengthened.

It is of interest that the strains that showed the greatest preference for alcohol in choice studies also showed the greatest resistance to the effect of injected alcohol in sleeping time studies. In enzyme studies (*9*) with C57BL/Crgl mice (the strain preferring alcohol) and BALB/cCrgl mice (the strain preferring water), the former showed a significantly higher alcohol dehydrogenase activity. However, the difference in hepatic enzyme activity seems too small to be the entire physiological basis for the difference in strain responses to alcohol. We found no significant difference between the two strains in their rates of ethanol metabolism. The explanation may be that the dose of alcohol used to induce sleeping was so large as to mask the slight difference in metabolic rates. Another factor which may influence metabolic degradation of alcohol is the rate of blood circulation. Even if a difference exists in the circulation of blood in these two strains, the brain-alcohol levels during the time period observed were identical, indicating that the brains of the two strains are differentially affected by the same level of alcohol.

It would appear that at least three phenomena are based on genetic differences in these two strains of mice: (i) the preference for alcohol, (ii) the level of liver alcohol dehydrogenase activity, and (iii) the sleeping time. This last phenomenon is shown to be due to brain sensitivity. Other studies have shown an increased behavioral tolerance to injected alcohol as a result of prolonged drinking, and a decreased tolerance with age in certain mouse strains (*5*, *8*). This suggests that the response to alcohol is influenced by environmental and developmental factors, as well as by inborn susceptibility.

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References and Notes

1. A. Arvola and O. Forsander, *Nature* **191**, 819 (1961); G. E. McClearn and D. A. Rodgers, *J. Comp. Physiol.* **54**, 116 (1961); J. Mardones, M. N. Segovia, D. A. Hederra, *Quart. J. Stud. Alc.* **14**, 1 (1953); E. Tobach, *ibid.* **18**, 19 (1957).
2. A. Casey, *ibid.* **21**, 208 (1960); H. Kalant, *Canad. J. Biochem. Physiol.* **41**, 2197 (1963); K.-H. Kiessling and K. Tilander, *Quart. J. Stud. Alc.* **22**, 535 (1961); M. Smith *et al.*, *J. Biochem.* **234**, No. 6, 1544 (1959).
3. H. W. Newman and J. Card, *J. Nerv. Ment. Dis.* **86**, 428 (1937); R. B. Forney and F. W. Hughes, *Clin. Pharmacol. Therap.* **4**, 619 (1963).
4. G. E. McClearn, *Proc. Int. Conf. Alc. Road Traffic* **3** (1962).
5. R. Kakihana, thesis, University of California, Berkeley, 1965.
6. K. D. Parker, C. R. Fontan, J. L. Yee, P. C. Kirk, *Anal. Chem.* **34**, 1234 (1962).
7. J. P. Guilford, *Fundamental Statistics in Psychology and Education* (McGraw-Hill, New York, 1956), p. 252.
8. R. Kakihana, G. E. McClearn, D. Treiman, unpublished data.
9. E. L. Bennett and M. Hebert, *Univ. Calif. Radiat. Lab. Quart. Rep.* No. 9208, April 1960.

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Cytochemistry of Synapses: Selective Staining for Electron Microscopy

Abstract. *Material at synapses and in some synaptic vesicles becomes selectively stained when glutaraldehyde-fixed rat brain that has not been treated with osmium is stained with phosphotungstic acid. The material stained at synapses is distinct from the adjacent unstained synaptic membranes and has cytochemical properties of protein. The specialized spatial arrangement exhibited by this synaptic material suggests its close involvement in synaptic function.*

The fine structural details of the specialized interneuronal synaptic contacts have traditionally been observed in brains fixed with osmium tetroxide (*1*). Fixation with an aldehyde (*2*) before exposure to the osmium reveals similar synaptic morphology (*3*) (Fig. 1). One distinguishing feature of the synapse in tissue fixed or stained with osmium is the presence of electron-opaque material associated with the presynaptic and postsynaptic membranes. However, there has been virtually no cytochemical characterization of this unknown cellular material that distinguishes synaptic sites from other apposing neuronal membranes. Although the synaptic material does not react with $KMnO_4$, Gray (*4*) has emphasized that enhanced electron-opacity of synaptic membrane components occurs when osmium-fixed brain is stained with phosphotungstic acid. The mechanism of this enhancement is unknown.

In this study, we have departed from the usual preparative procedure for electron microscopy: rat brain was fixed by glutaraldehyde perfusion and stained with alcoholic phosphotungstic acid (PTA) without being exposed to osmium tetroxide. Although membranes are not positively stained by this procedure, electron-opacity is pronounced at synaptic sites (Fig. 2). In fact, electron-opacity in the neuropil is, with one exception, observed only at synaptic sites. Four fine structural details are demonstrable: (i) a highly electron-opaque band beneath the postsynaptic membrane, parallel to the synaptic cleft, and 150 to 250 Å wide; (ii) several clumps of less intense, electron-opaque material projecting from the area of the presynaptic membrane into the terminal bouton; (iii) a discontinuous electron-opaque line (50 to 150 Å) occupying the synaptic cleft separated

from each of the other stained elements by a 60- to 80-Å gap; (iv) small fibrillar tufts of low electron-opacity subjacent to the subsynaptic band. The sole exception to the exclusive selective staining of synapses in the neuropil with glutaraldehyde-PTA is the opacity developed in certain isolated, 400-Å-wide presynaptic spheres (Fig. 2). Elsewhere, nuclear and nucleolar particles are also densified by this procedure.

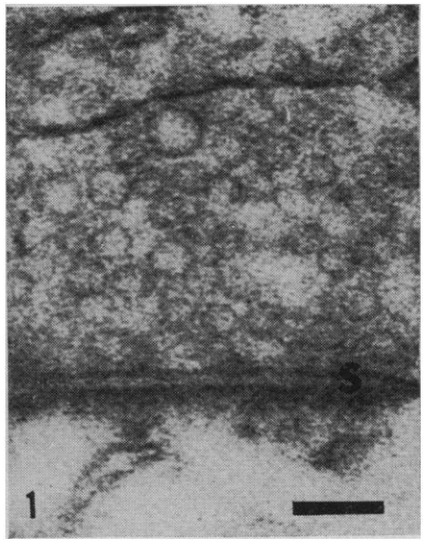
In evaluating the generality of the selective synaptic staining, we examined synapses in a number of areas including the hypothalamus, cerebellar and cerebral cortex, olfactory bulb (*5*), superior cervical ganglion, and the nerve terminations on adrenal medullary cells (*6*) after they had been perfused with glutaraldehyde and stained with alcoholic PTA. In each case (*7*) synaptic sites exhibited essentially the same four details that are described above, including the presynaptic, electron-opaque spheres.

Synapses were examined in the paraventricular region of the rat hypothalamus fixed with glutaraldehyde and stained by various procedures: aqueous PTA, aqueous PTA before or after treatment with osmium tetroxide, alcoholic PTA before or after osmium tetroxide, alcoholic PTA or alcoholic uranyl acetate without exposure to osmium tetroxide, and glutaraldehyde fixation only. We also tried to stain thin Maraglas sections of brain treated with glutaraldehyde and osmium by bleaching (*8*) them and subsequently floating them on either aqueous or alcoholic solutions of PTA or uranyl acetate. In tissue treated only with glutaraldehyde, no electron opacity suggestive of synaptic complexes was seen. The selective staining of synaptic material seen in tissue stained with alcoholic PTA was not observed in that stained by any of the other procedures.

Correlation of the hypothalamus treated with glutaraldehyde and PTA with glutaraldehyde-osmium stained tissue was attempted. We found that exposure of thin Maraglas sections of glutaraldehyde-PTA tissue to osmium tetroxide with or without conventional lead staining did not increase the density of the membranes enough to permit comparison of adjacent thin sections. On the other hand, staining of thin, bleached sections (*8*) with alcoholic PTA did not reveal the selective staining of synaptic material. Therefore, we compared the two by estimating the frequency of synapses per unit area of thin sections taken from adjacent blocks of paraventricular hypothalamus and found that most, if not all, of the synapses seen in osmium preparations are also stained by alcoholic PTA.

However, brain fixed primarily with osmium or stained with osmium after glutaraldehyde fixation does not selectively reveal the four synaptic details even when followed by staining with alcoholic PTA (*4*) since all membranes and other synaptic components already show some electron opacity after treatment with osmium. Moreover, these details are revealed far more strikingly in the absence of osmium tetroxide than when PTA is applied after osmium (*4*). After treatment with osmium tetroxide, the electron-opacity and size of the individual components, particularly cleft and presynaptic material, stained by alcoholic PTA appears markedly attenuated. That membranes are not stained by our glutaraldehyde-alcoholic PTA procedure (*4*) indicates that the special synaptic material is distinct from the membranes. The apparently wider synaptic cleft of the PTA-stained synapses is accounted for by the lack of staining of the presynaptic and postsynaptic membranes.

Further comparison of the two preparative procedures indicates that the isolated presynaptic spheres that stain with PTA correlate in size and position either with the electron-opaque central core of the granular or "dense-core" synaptic vesicles or with certain agranular vesicles seen after conventional tissue preparation (*4*, *9*). In the paraventricular hypothalamus, dense vesicle "cores" of similar size and cellular frequency were seen when tissue routinely treated with osmium was compared with that treated with glutaraldehyde and PTA. The chromaffin granules of adrenal medullary cells also were made opaque with this PTA stain. On the other hand, in areas of rat brain, such as cerebral or cerebellar cortex, which after osmium-fixation rarely exhibit typical dense-core vesicles, spherical densities of the same size as those in the



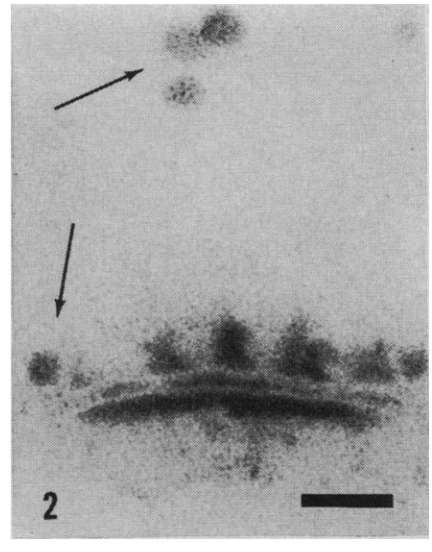


Fig. 1. Electron micrograph of axodendritic synapse in rat paraventricular hypothalamus fixed by perfusion with 5 percent glutaraldehyde (phosphate buffered, *p*H 7.4); this block was stained for one hour with 1 percent buffered osmium tetroxide (*p*H 7.4) before being dehydrated in ethanol and embedded in Maraglas. Silver thin-sections were cut on a Sorvall MT-2 microtome and examined in an RCA EMU 3-F electron microscope, without any additional contrast-enhancing procedure. An axon terminal filled with agranular vesicles is above the horizontally oriented synaptic cleft (*S*). Fibrillar material is prominent beneath the subsynaptic membrane below (scale, 1000 Å). Fig. 2. Electron micrograph of axodendritic synapse in rat paraventricular hypothalamus. Tissue prepared as in Fig. 1, except that there was no exposure to osmium. Instead, the dehydrated block was treated with 1 percent PTA (in absolute alcohol) for 1 hour. The synapse extends horizontally and is oriented as in Fig. 1 with nerve terminal above and dendrite below. Except for the subsynaptic component, virtually none of the other components of the synapse shown here can be seen without PTA (compare with Fig. 1). On the other hand, membranous structures, including most synaptic vesicles, are not seen without osmication. Gaps or "pores" can be seen in the stained cleft material. The arrows indicate the isolated electron dense spheres within the nerve ending (scale, 1000 Å).

hypothalamus are commonly seen after staining with PTA; thus, all agranular vesicles seen after osmium preparation may not be chemically identical. The dense-core vesicles have been associated with storage sites of certain biogenic amines (*10*). However, staining with PTA is not dependent upon the reduction of a metallic oxidant as are the histochemical reactions used to demonstrate catechols and indoles (*11*). Therefore, the staining of vesicles with PTA would not necessarily reflect their content of biogenic amines. The staining of vesicle cores with PTA could indicate that a matrix material of similar chemical reactivity is shared by certain agranular vesicles and also possibly by the dense-core synaptic vesicles. Although no correlation between the PTA-stained vesicle cores and the PTA-stained presynaptic tufts or projections (*4*) has been demonstrated, their possession of similar chemical affinity for PTA suggests a possible relationship.

The dense material associated with specialized intercellular junctions (*12*) in nonneuronal tissue also stains selectively with alcoholic PTA in glutaraldehyde-fixed tissue. In junctions of the macula adherens type (*12*) between neighboring ependymal cells and adjacent adrenal medullary cells, there is an electron-opaque material resembling the electron-opaque band subjacent to the postsynaptic membrane in both form and staining intensity. At the nonneuronal intercellular junctions, the material stained by PTA is bilaterally symmetrical, as if in mirror images. Thus, only at synaptic sites is the stained material arranged in a polarized, oriented fashion; this fact suggests that the function, if not the composition, of the nonneuronal specialized contact zones is different (*1, 4*).

Phosphotungstic acid has a high molecular weight and has been used as a precipitating agent in the isolation of basic amino acids (*13*). The staining of collagen by PTA has been related to its binding to specific basic amino acids (*14*). The special material at synapses may be a protein containing a high proportion of basic amino acids such as lysine, histidine, and arginine. Basic proteins are also found associated with nuclear DNA (*15*) and may account for the affinity of the nuclear material for PTA. To analyze which broad classes of cellular macromolecular components might be causative in the alcoholic-PTA staining of synpatic material, we treated 80-μ frozen sections of glutaraldehyde-fixed paraventricular hypothalamus with deoxyribonuclease, ribonuclease (*16*), pepsin, trypsin, and testicular hyaluronidase. Of these treatments, only proteolysis removed the PTA-stained material. The synaptic vesicles and membranes of tissue stained with osmium after proteolytic digestion appeared well preserved. Thus, the proteolytic digestions selectively remove the material stained at synapses by alcoholic PTA without disturbing membranous components. Although this type of cytochemical experimentation cannot be taken as definitively identifying the PTA-reactive synaptic material, the involvement of protein is strongly suggested.

Our results indicate that synaptic sites have, in addition to their membranes, a component distinguished by its affinity for PTA. This material, which may be proteinaceous, is in a special spatial orientation with respect to interneuronal transmission. The electron-microscopic staining procedure which reveals this material to be distinct from synaptic membranes offers an additional approach to the cytochemical analysis of the synapse. The further characterization of this material as to structural and enzymatic composition, functional turnover, and chemical binding properties may clarify its significance in the formation and function of synapses.

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References and Notes

1. S. L. Palay, *J. Biophys. Biochem. Cytol.* **2** suppl., 193 (1956); *Exp. Cell Res. Suppl.* **5**, 275 (1958); ——— and G. E. Palade, *J. Biophys. Biochem. Cytol.* **1**, 68 (1955).
2. D. Bodian, *Bull. Johns Hopkins Hosp.* **114**, 13 (1964); U. Karlsson and R. L. Schultz, *J. Ultrastruct. Res.* **12**, 160 (1965).
3. T. S. Reese and M. W. Brightman, *Anat. Rec.* **151**, 492 (1965).
4. E. G. Gray and R. W. Guillery, *Int. Rev. Cytol.* **19**, 111 (1966).
5. K. H. Andres, *Z. Zellforsch.* **65**, 530 (1965).
6. R. E. Coupland, *J. Anat.* **99**, 231 (1965); L. G. Elfvin, *J. Ultrastruct. Res.* **12**, 263 (1965).
7. Some synapses in the external plexiform layer of the olfactory bulb are unique in having synaptic vesicles on both presynaptic and postsynaptic sides (*3, 5*). At these sites the details of the PTA-stained material vary from the present description.
8. V. Marinozzi, *J. Roy. Microscop. Soc.* **81**, 141 (1963).
9. D. E. Wolfe, in *Abstr. Ann. Meeting Amer. Soc. Cell Biol.*, 1st, p. 228 (1961); A. Pelligrino de Iraldi, H. F. Duggan, E. de Robertis, *Anat. Rec.* **145**, 521 (1963); F. E. Bloom, G. K. Aghajanian, R. J. Barnett, in *Biochemistry and Pharmacology of the Basal Ganglia*, E. Costa, L. J. Cote, M. D. Yahr, Eds. (Raven Press, New York, in press).
10. V. P. Whittaker, *Progr. Biophys. Mol. Biol.* **15**, 39 (1965); E. de Robertis, A. Pellegrino de Iraldi, G. R. Arnaiz, L. Salganicoff, *J. Neurochem.* **9**, 23 (1962); G. K. Aghajanian and F. E. Bloom, *Science* **153**, 308 (1966).
11. R. E. Coupland, A. S. Pyper, D. Hopwood, *Nature* **201**, 1240 (1964); J. G. Wood and R. J. Barrnett, *J. Histochem. Cytochem.* **12**, 197 (1964); J. G. Wood, *Nature* **209**, 1131 (1966); F. E. Bloom and R. J. Barrnett, *ibid.* **210**, 599 (1966).
12. M. G. Fahrquhar and G. E. Palade, *J. Cell Biol.* **17**, 375 (1963).
13. J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids* (Wiley, New York, 1961), pp. 651–53.
14. C. R. Zobel and M. Beer, *Int. Rev. Cytol.* **18**, 363 (1965); K. Kühn, *Leder* **9**, 217 (1958); F. O. Schmitt, C. E. Hall, M. E. Jackus, *J. Appl. Phys.* **16**, 263 (1945).
15. E. W. Johns and J. A. Butler, *Biochem. J.* **84**, 436 (1962).
16. Deoxyribonuclease and ribonuclease were furnished by Dr. N. R. Morris, Department of Pharmacology, Yale University School of Medicine, New Haven, Conn., who collaborated in the experiments with these enzymes.
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Open-Field Behavior in Mice: Evidence for a Major Gene Effect Mediated by the Visual System

Abstract. *In segregating F_2, F_3, and F_4 generations, albino mice had lower activity and higher defecation scores than pigmented animals when tested in a brightly lighted open field. These differences persisted when members of an F_5 generation were tested under white light, but largely disappeared under red light. Thus it was concluded that there is a major gene effect on the quantitative traits of open-field activity and defecation which is mediated by the visual system and that albino mice are more photophobic than pigmented mice under conditions of bright illumination.*

Merrell (*1*) has recently reviewed studies reporting the effects of single genes on behavior and has suggested that the study of gene substitutions, one or a few at a time, may provide information not only about the genetics of a quantitative behavioral trait, but about the trait itself. In a recent quantitative