Syntheses and *In-vitro* Evaluation of Tetrahydroaminoacridine (THA) Based Analogues as High Affinity Choline Transporter (HAChT) Imaging Probe

[1]Mohammad Anwar-Ul Azim, [2]Takashi Kozaka, [2]Izumi Uno, [2]Daisuke Miwa, [2]Yoji Kitamura, [3]Kazuma Ogawa, [2]Kahuhiro Shiba

[1]National Institute of Nuclear Medicine and Allied Sciences, BAEC, BSMMU, Shahbagh, Dhaka.
[2]Division of Tracer Kinetics, Advanced Science Research Center, Kanazawa University, Japan.
[3]Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Japan.

Correspondence: Dr. Mohammad Anwar-Ul Azim, Senior Scientific Officer, National Institute of Nuclear Medicine & Allied Sciences (NINMAS), Bangladesh Atomic Energy Commission (BAEC), BSM Medical University Campus, Block-D, 8th Floor, Shahbagh, Dhaka-1000. E-mail: anwarri79@gmail.com

ABSTRACT

Introduction: **In cholinergic neurons, high affinity choline uptake (HACU) by the high affinity choline transporter (HAChT) is a rate-limiting and regulatory step for the synthesis of Acetylcholine (Ach).Thus, HAChT appear to be a relatively specific presynaptic marker for cholinergic neurons in Alzheimer's disease.**

Objectives: **The principle objective of the study is to check the affinity of tetrahydroaminoacridine (THA) derivatives for HAChT. Another objective of the research work is to clarify whether the hemicholinium-3 (ChT inhibitor) and HACU enhancer molecules share the same binding sites or not.**

Materials and Methods: The inhibition activities of tacrine, the 2,3-dimethylfuran derivative of tacrine (DMTA) and their corresponding 2-oxo-1-pyrrolidineacetyl derivatives, namely PTAA and MKC-231 were measured by displacement of a typical HAChT antagonist [^{3}H]HC-3 in rat cerebral membrane. The percentage of inhibition against the binding of [^{3}H]HC-3 to HAChT were calculated using GraphPad Prism v4 software.

Results: **Hemicholinium-3 showed affinity for HAChT (IC_{50} = 20 nM) in the *in vitro* binding assay. A very insignificant inhibition activity (IC_{50} = 1000 nM) of Tacrine was revealed. The newly synthesized tacrine derivatives, DMTA and PTAA did not show any affinity for HAChT. Although MKC-231 was reported to enhance cholinergic activity at synaptic terminals, it did not show any affinity for the HAChT in [^{3}H]HC-3 binding assay.**

Conclusion: In vitro **[^{3}H]HC-3 binding assay revealed no affinity of MKC-231, tacrine and its corresponding2-oxo-1-pyrrolidineacetate derivative towards HAChT. So, it is worthy to develop radiolabeled HC-3 derivatives with high affinity for HAChT, which can diffuse the BBB, to enable the *in vivo* investigation of HACU system.**

Key Words: **Tacrine, High Affinity Choline uptake, MKC-231, Cholinergic Neurotransmission**.

INTRODUCTION

The high-affinity choline uptake system located in peripheral and central cholinergic nerve terminals plays a regulating and rate-limiting role in the intraneuronal synthesis of acetylcholine (ACh) (1, 2). Choline is transferred into the cell by the high-affinity choline transporter where it reacts with acetyl CoA in the presence of the enzyme choline acetyltranferase (ChAT) to form acetylcholine. Brain synaptosome studies demonstrate two carrier-mediated transport systems for choline uptake (3, 4). At high concentrations, choline is transported primarily by a low-affinity Na^+-independent system that is inhibited by hemicholinium-3 (HC-3) (5) with a high K_i of approximately 50 μM. This system is thought to be ubiquitously present in cells and to be required for phosphatidylcholine synthesis. At low concentrations choline is transported by a high-affinity, Na^+-dependent system that is inhibited by HC-3 with a low K_i of 10–100 nM. The high-affinity system is supposed to be present specifically in cholinergic neurons, because a substantial proportion of choline is converted to acetylcholine only when taken up through the high-affinity system (3, 4). The proposal that the high-affinity choline transport system is unique to cholinergic neurons is supported by the selective loss of the high-affinity choline uptake following depletion of cholinergic terminals in a variety of denervation studies (4). Choline uptake is generally believed to be the rate-limiting step in acetylcholine synthesis (3, 4). In addition, the high-affinity choline uptake is regulated by neuronal activity

activity also regulate acetylcholine synthesis. In Alzheimer's disease, cholinergic neurons selectively degenerate. Consistent with the above hypothesis, the high-affinity choline transporter is reduced in Alzheimer's disease (7).

Pathogenesis of AD is highly complex. Researchers around the globe have proposed several hypotheses for explaining the mechanism of AD development. Among the hypotheses, cholinergic hypothesis has been widely approved and the most characteristic abnormality associated with AD is the decline of cholinergic neurotransmission. Deficiency in high affinity choline uptake (HACU) by the high affinity choline transporter (HAChT) and the loss of vesicular acetylcholine transporter (VAChT), are two characteristic neurochemical changes in AD. High affinity choline uptake transporter can serve as a useful marker of the functional status of the cholinergic presynaptic terminals and is known to be associated with a variety of diseases, such as Parkinson's disease, Alzheimer's disease, coronary heart diseases, tumor cancers (8, 9). Thus, choline transporter systems provide attractive targets for the development of PET biomarkers to probe brain, heart, and cancer diseases.

Tacrine (9-amino-1,2,3,4-tetrahydroacrydine, THA, commercial name: Cognix), is a well known acetylcholinesterase (AChE) inhibitor, which produces clinically significant improvement in some cognitive deficits observed in AD patients by increasing the synaptic availability of Ach by inhibiting the AChE-mediated degradation of ACh to choline and acetate. But efficacies of this drug are limited and commonly associated with gastrointestinal side effects during long treatment period (10). Besides this, there is a deficiency of choline uptake in AD patients. Hence, activation of the presynaptic cholinergic function by improving the reduced HACU is another better approach for the treatment of AD.

Although, THA has no effect as HACU enhancer, THA contained 4-aminopyridine (4 - AP) as a partial structure, which is already known to increase HACU and 1-nitrogen atom of 4-AP, also corresponds to the quaternary amine of ACh(Figure 1).In 1995, Chaki, H., et al showed acylation of THA leads to the synthesis of novel choline uptake enhancers which improve the reduced HACU and also that some of the structural features of N-acyl derivatives of THA resembles acetylcholine. But due to this structural modification THA lose its activity as AChE inhibitors (11).

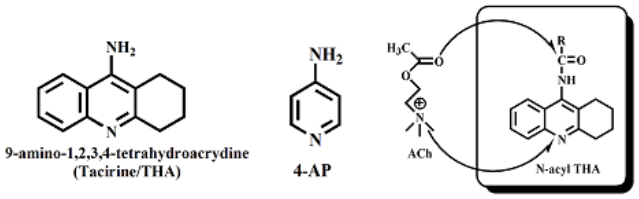


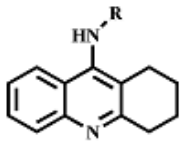
Figure 1. Chemical structure of THA, 4-AP, Ach and N-acyl THA

Taking into the consideration, this special characteristic feature of N-acyl derivatives of THA as HACU enhancer and the role of HAChT to enhance HACU, we have synthesized tacrine, the 2,3-dimethylfuran derivative of tacrine (DMTA) and their corresponding 2-oxo-1-pyrrolidineacetyl derivatives, namely PTAA and MKC-231 and evaluated these compounds through *in vitro* [^{3}H]-Hem-3 binding assay. The objectives of the evaluation of these THA derivatives are as follows: (a) to check the affinity of MKC-231, Tacrine and the newly synthesized THA derivatives (DMTA, PTAA) for HAChT, (b) to find out the possible explanation of mode of action (MOA) of HACU enhancer, and (c) to make a possible clarification of the fact that whether thehemicholinium-3 (ChT inhibitor) and HACU enhancer molecules share the same binding sites or not.

MATERIALS AND METHODS

Chemical Syntheses: The syntheses of Tacrine, the 2,3-dimethylfuran derivative of Tacrine (DMTA) and their corresponding 2-oxo-1-pyrrolidineacetyl derivatives, namely PTAA and MKC-231, were accomplished with the following schemes:

Scheme 1: Synthesis of tacrine (THA) and 2-oxo-1-pyrrolidineacetyl derivative of tacrine, namely PTAA (Figure 2).



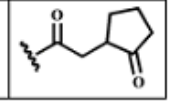
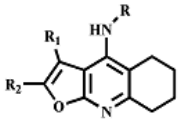
Compounds	R
Tacrine (THA)	H
PTAA	

Figure 2. Chemical structure of Tacrine and PTAA

Scheme 2: Replacement of the phenyl ring on the left part of the tricyclic structure of tacrine with a furan ring and synthesis of it's 2-oxo-1-pyrrolidineacetyl derivative (Figure 3)



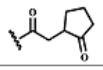
Compounds	R_1	R_2	R
DMTA	Me	Me	H
MKC-231	Me	Me	

Figure 3. Chemical structure of DMTA and MKC-231

Preparation of rat cerebral and liver membranes

Animal experiments were performed in compliance with the guidelines for the care and use of Laboratory Animals at the Takara-machi Campus of Kanazawa University. Brain homogenate of rats were prepared with the slight modification of previously described protocol (12). Sprague-Dawley rats (8 weeks, male, 250–300 g) cerebrum or liver were homogenized in ice-cold 0.32 M sucrose with a Teflon-glass homogenizer. The homogenate was centrifuged at 1000g at 4 ^{o}C for 10 min. The resulting precipitate was removed and the supernatant was centrifuged at 20,000 g at 4 ^{o}C for 20 min. This crude mitochondrial pellet was re-suspended in 20 vols. of ice-cold distilled water and dispersed with the Teflon homogenizer with 1000 rpm and the homogenate centrifuged at 8000 g run for 20 min. The supernatant and buffy coat were collected and pelleted at 48,000 g for 20 min. The pellet was washed 4 times in 20 vols. of 50 mM Glycylglycine buffer (pH 7.8) containing 200 mM NaCl by resuspending via Teflon homogenizer. Then the homogenate was centrifuged at 55,000 g for about 15 min and the resulting pellet was again resuspended in 50 mM Glycylglycine buffer (pH 7.8) containing 200 mM NaCl with a Teflon homogenizer (Figure 4).

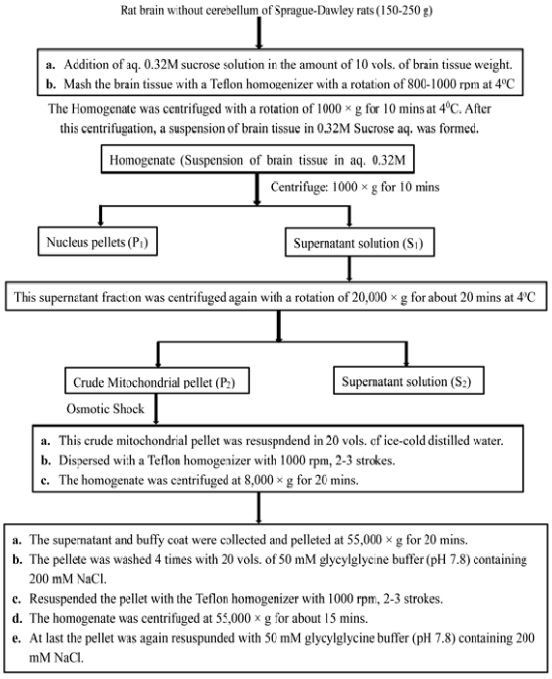


Figure 4. Flow chart for the homogenate reparation of rat cerebral and liver membranes.

In vitro HAChT [^{3}H]Hemicholinium--3 binding assay

[^{3}H]Hemicholinium-3 (K_d = 19.1 nM) (20) was used as a specific radioligand for the HAChT receptor. Rat cerebral membranes were added to each ice-cold assay tube containing [^{3}H]HC-3 and the displacing ligands at various concentrations ($1.0 \times 10^{-9} – 10^{-4}$) in 50 mM Glycylglycine buffer (pH 7.8) containing 200 mM NaCl in quadruplicate. After the addition of tissues in an ice bath, each reaction mixture in the tube was incubated at 25^{o}C for 30 min. The incubation was terminated by putting tubes into ice-cold water followed by immediate filtration using a cell harvester through glass-microfiber filters (Whatman, GF/B), which were presoaked in 0.5% (v/v) polyethyleneimine for one hour to reduce non-specific binding. The filters were washed with 50 mM Tris-HCl buffer (pH=7.8), and the radioactivity was counted with the liquid scintillation counter (Aloka, LSC-5100).

RESULTS

Chemistry: The cyclization of 2-Amino benzonitrile (1) and 2-Amino-4, 5-dimethyl-3-furancarbonitrile (2) with cyclohexanone by means of $ZnCl_2$ in toluene, furnished tacrine (3) and DMTA (4), respectively (21)(Figure 5).

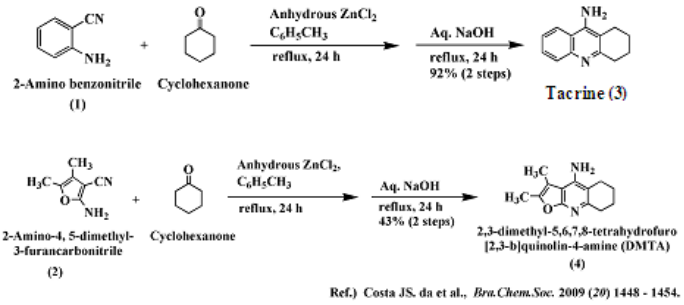


Figure 5. Scheme for the syntheses of tacrine and DMTA

The acylation of Tacrine (3) and DMTA (4) with methyl 2-oxo-1-pyrrolidineacetate furnished PTAA (5) and MKC-231 (6) with the yield of 79% and 65% respectively (Figure 6).

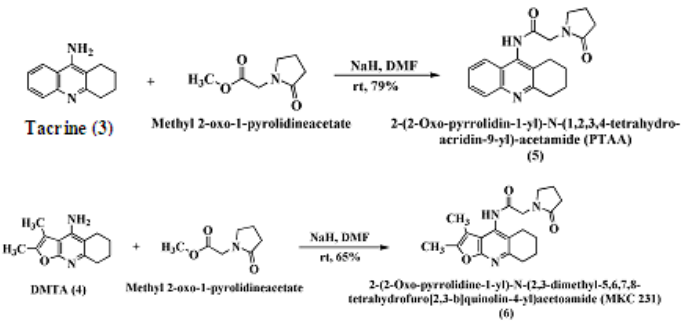


Figure 6. Scheme for the syntheses of PTAA and MKC-231

In vitro HAChT [^{3}H]Hemicholinium-3 ([^{3}H]HC-3) binding assay

The inhibition activities of tacrine (THA), MKC-231 and the two newly synthesized tacrine derivatives, namely DMTA and PTAA were measured by displacement of a typical HAChT antagonist [^{3}H]HC-3 (Dissociation constant: K_d = 19.1 nM) (20) in rats cerebral membrane. The percent inhibition against the binding of [^{3}H]HC-3 to HAChT were calculated using GraphPad Prism v4 software (Figure 7). HC-3 showed the highest affinity for HAChT(IC_{50} = 20nM). Tacrine (THA) showed very insignificant inhibition activity (IC_{50} = 1000 nM).Both the 2, 3-dimethylfuran derivative of tacrine, DMTA and 2-oxo-1-pyrrolidineacetyl derivative of tacrine, PTAA showed no affinity for HAChT.MKC-231 also did not show any affinity for the HAChT in [^{3}H]Hemicholinium-3 binding assay.

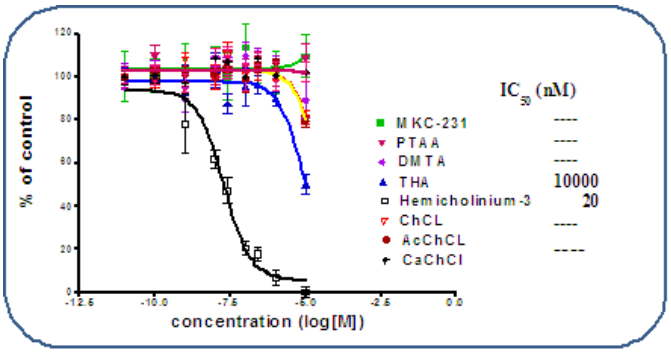


Figure 7. *In vitro* HAChT [^{3}H] Hemicholinium-3 binding assay

DISCUSSIONS

High affinity choline uptake (HACU) system is located especially in presynaptic cholinergic nerve terminals. HACU by the HAChT is found to be a regulating and rate-limiting step in the synthesis of ACh. Hence, a ligand with a high affinity for HAChT would be an ideal marker for the study of the state of cholinergic presynaptic terminals.Hemicholinium-3 is an indirect acetylcholine antagonist, because it decreases the synthesis of acetylcholine by inhibiting the reuptake of choline by the high-affinity choline transporter (ChT). As HC-3 is a quaternary ammonium salt (Figure 8), it cannot cross BBB and radiolabeled quaternary ammonium HC-3 derivatives cannot be used for in vivo visualization of the HACU transporter. MKC-231, 2-(2-Oxo-pyrrolidine-1-yl)-*N*-(2,3-dimethyl-5,6,7,8-tetrahydrofuro [2, 3-b]quinolin-4-yl) acetoamide, has been reported to enhance HACU in hippocampal synaptosomes in *in vitro* treatment and ameliorates learning deficits in AF64A-treated rats with a single oral administration (22). Although MKC-231 potentially enhances cholinergic activity at synaptic terminals and improves learning impairment observed in AF64A-treated rats and mice, no precise HACU

enhancement mechanism has been elucidated. Acylation of THA, an AChE inhibitor, led to the synthesis of novel choline uptake enhancers, which improves the reduced HACUin AD. In the process THA lose its activity as AChE inhibitors (11).

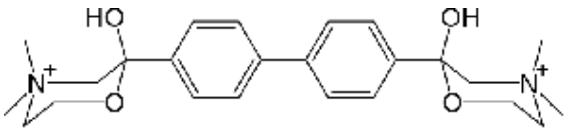


Figure 8. Chemical structure of Hemicholinium-3 (HC-3)

In the effort of developing HAChT imaging probe we have evaluated tacrine, the 2,3-dimethylfuran derivative of tacrine (DMTA) and their corresponding 2-oxo-1-pyrrolidineacetyl derivatives, namely PTAA and MKC-231 through *in vitro* [^{3}H]Hemicholinium-3 ([^{3}H]HC-3)binding assay to investigate their affinity for HAChT. From the *in vitro* binding assay, it was revealed that only hemicholinium-3 expressed high affinity for HAChT(IC_{50} = 20 nM).Tacrine (THA) showed somedegree of inhibition activity (IC_{50} = 1000 nM), but it is very insignificant compared to the inhibition activity of hemicholinium - 3 (IC_{50} = 20 nM). Both the 2,3-dimethylfuran derivative of tacrine, DMTA and 2-oxo-1-pyrrolidineacetyl derivative of tacrine, PTAA showed no affinity for HAChT. Although, MKC-231, is known to enhance HACUand increase the synthesis and release of ACh in *in vitro* and *in vivo* studies (23), it did not show any affinity for the HAChT in [^{3}H] Hemicholinium-3 binding assay. Other inhibitors like choline chloride (ChCl), Acetylcholine chloride (AChCl), Carbamylcholine chloride (CaChCl) showed no binding affinity for high affinity choline transporter (ChT).

As no inhibitors (except Hem-3), MKC-231, THA and the synthesized THA derivatives did not exhibit any inhibition towards HAChT, so it is worthy to search for the new molecules having high affinity for choline transporter (HAChT) for the extensive study of HACU.

CONCLUSION

In a effort to develop tacrine and its 2-oxo-1-pyrilidineacetyl derivatives as HAChT imaging probe, tacrine, DMTA (2, 3-dimethylfuran derivative of tacrine) and their corresponding 2-oxo-1-pyrrolidineacetyl derivatives, namely PTAA and MKC-231, was synthesized and the binding affinities of these derivatives for HAChT were evaluated through *in vitro* HAChT [^{3}H]HC-3 competitive binding assay. No affinity towards HAChT of the synthesized compounds was revealed from the *In vitro* binding assay.

Hemicholinium-3 is a competitive inhibitor of the HACU system and it is worthy to do further research works for the development of radiolabelled HC-3 derivatives with high affinity for HAChT, which can diffuse the BBB, to enable the *in vivo* investigation of HACU system. It is also worthy to do more extensive review to develop an effective *in vitro* HAChT [^{3}H]HC-3 competitive binding assay protocol and the protocol for the preparation of brain homogenate, used in the *in vitro* HAChT [^{3}H]HC-3 competitive binding assay.

REFERENCES

1. Smart LA. Synthesis and biological activity of a 2-bromoethylamine (mustard) derivative of hemicholinium-3 and hemicholinium-15. J Med. Chem1983; 26: 104-107.
2. Gilissen C, de Groot T, Bronfman F, van Leuven F, Verbruggen A. M, Bormans G. M. Evaluation of ^{18}F–FA-4 and ^{11}C–pipzA-4 asRadioligands for the In Vivo Evaluation of theHigh-Affinity Choline Uptake System. J Nucl. Med 2003;269-275.
3. Haga, T. Synthesis and release of [^{14}C] acetylcholine in synaptosomes. J. Neurochem

1971;18**:**781–798.

4. Kuhar MJ, Murrin LC. Sodium-dependent, high-affinity choline uptake. J. Neurochem 1978;30:15–21.
5. Happe HK, Murrin LC. High-affinity choline transport sites: use of [^{3}H] hemicholinium-3 as a quantitative marker. J. Neuro chem 1993; 60**:** 1191–1201.
6. Murrin LC, Kuhar MJ. Activation of high-affinity choline uptake *in vitro* by depolarizing agents. Mol. Pharmacol1976; 12**:**1082–1090.
7. Pascual J, Fontán A, Zarranz JJ, Berciano J, Flórez J, Pazos A. High-affinity choline uptake carrier in Alzheimer's disease: implications for the cholinergic hypothesis of dementia. Brain Res 1991; 552**:**170–174.
8. Hara T, Bansal A, DeGrado TR. Choline transporter as a novel target for molecular imaging of cancer. Molecular Imaging 2006; 5: 498-509.
9. Gao M, Miller MA, De Grado TR, Mock BH, Lopshire JC, Rosenberger JG, Dusa C, Das MK, Groh WJ, Zipes DP, Hutchins GD, Zheng QH. Evaluation of [^{11}C]hemicholinium-15 and [^{18}F]hemicholinium-15 as new potential PET tracers for the high-affinity choline uptake system in the heart. Bioorg. Med. Chem 2007; 15(*3*): 1289-1297.
10. Terry AV Jr, Buccafusco JJ. The cholinergic hypothesis of age and Alzheimer's disease related cognitive deficits: Recent challenges and their implications for novel drug development. J PharmacolExpTher2003; 306: 821-827
11. Chaki H, Yamabe H, Sugano M, Morita S, Bessho T, Tabata R, Saito KI, Egawa M, Tobe A, Morinaka Y. Design and synthesis of 4-acylaminopyridine derivatives: Novel high affinity choline uptake enhancer I[1)]. Bioorganic and Medicinal Chemistry Letters 1995; 5 (*14)*: 1489-1494.
12. Sandberg K, Coyle JT. Characterization of [^{3}H]Hemicholinium-3 binding associated with Neuronal choline uptake sites in rat membranes. Brain research 1985; 348: 321-330.
13. Quirion R. Characterization and autoradiographic distribution of Hemicholinium-3 high affinity choline uptake sites in mammalian brain. Synapse 1987; 1: 293-303.
14. Costa JS da, Pisoni DS, Silva CB da, Petzhold CL, Russowsky D, Ceschi MA. Lewis Acid Protonated Friedlander Condensation Reactions between Anthranilonitrile and Ketones for the Synthesis of Tacirine and its Analogues. J. Braz. Chem. Soc. 2009; 20 (9): 1448-1454.
15. Bessho T, Takashina K, Tabata R, Ohshima C, Chaki H, Yamabe H, Egawa M, Tobe A, Saito K. Effect of the novel high affinity choline uptake enhancer 2-(2-oxopyrrolidin-1-yl)-*N*-(2,3-dimethyl-5,6,7,8-tetrahydrofuro[2,3-b]quinolin-4-yl)acetoamide on deficits of water maze learning in rats. Arzneim.-Forsch/Drug Res 1996; 46(*1*): 369-373.
16. Takashina K, Bessho T, Mori R,EguchiJ, Saito K. MKC-231, a choline uptake enhancer: (2) Effect on synthesisand release of acetylcholine in AF64A-treated rats. J Neural Transm2008; 115: 1-27-1035.