TUDOMÁNYOS KOLLOKVIUM TÉZISEI

Serotonergic innervation of the avian ventral tegmental area

A madár area ventralis tegmentalis (Tsai) neurocitokémiai, pályakapcsolati és elektronmikroszkópos vizsgálata, különös tekintettel a serotonint tartalmazó idegelemekre

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I. Summary of the scientific background

The area ventralis tegmentalis of Tsai (VTA), a midbrain monoaminergic cell group, is thought to play a role in memory formation, aversive/ addictive behaviours and stress related visceral responses. It is also responsible for the processing of a hedonistic component in certain forms of learning and memory, while representing an essential link within the brain reward cycle.

In mammals, the VTA and the raphe nuclei, together with the nucleus accumbens, are considered to be the main subcortical relays of the brain reward circuit, all possessing reciprocal connections with the medial prefrontal cortex, mPFC. This cortical area plays an essential role in the regulation of emotional changes and certain aspects of learning and memory. Forming part of the brain reward circuit, the ventral aspect of mPFC also is implicated in the generation of addictive behaviours.

The avian pallial structures comprise the hyperpallial areas (formerly, hyperstriatal layers) where the rostral Wulst, on the basis of its connectivity and neuronal composition, is thought to be homologous to a part of the mammalian ventromedial PFC. Therefore our aim was to find a direct Wulst-VTA neuronal loop to shed light to the hitherto unknown connections as well as to further support our hypothesis of the rostral Wulst being equivalent to a part of the „avian prefrontal cortex".

Although there has been substantial progress concerning information processing at the single cell and molecular levels in the VTA, our knowledge of its overall afferent connection in the avian brain is sparse.

The avian VTA is located in the vicinity of the emerging oculomotor nerves, forming part of the A10 dopaminergic cell group. The brainstem monoaminergic system of the domestic chicken shows reasonable similarity to that of the mammalian brain, therefore our aim was to detect whether such connections, preferably with an identical chemical nature, exist in the avian brainstem.

Afferents to the avian VTA, similar to those in the mammalian CNS, stem from the limbic forebrain, including the nucleus accumbens, diagonal band of Broca, septal nuclei, bed nucleus of the stria terminalis, hippocampal complex. The parvocellular medial striatum, arcopallium and the avian prefrontal cortex are also though to be interconnected with the VTA.

Previous studies in the rat brain have described that VTA neurones project to the dorsal raphe nucleus (DRN) with a certain contingent of the axons being GABAergic. Also, a reciprocal mainly

inhibitory projection, colocalizing serotonin (5HT) and GABA, from the DRN has been verified to reach the dopaminergic neurones of the VTA. Hence, 5HT action within the VTA enhances the release of dopamine in the nucleus accumbens, a major target of midbrain dopaminergic efferentation. Thus suggesting a direct synaptic action of 5HT afferents upon the dopaminergic VTA neurones.

Similar to that observed for the mammalian brain, a major serotonergic projection also is apparent from a mesopontine complex (nucl. linearis caudalis), partially terminating in the vicinity of dopaminergic neural somata, as seen in the light microscope.

Since the accurate identification of the avian mesopontine serotonergic (i.e. raphe) nuclei is still questionable, to prove the existence of a similar loop would be of great importance.

In order to describe the subdivisions, connectivity and the neurotransmitter content of the VTA, together with its electron microscopical structure, enzyme histochemistry, preembedding immunocytochemical techniques, as well as, anterograde and retrograde tract tracing methods were employed.

II. Aims of the study

1. The wiring of the monoaminergic nuclei shows substantial similarities when studied in the chick or rat brain, however, the avian PFC-ACC-VTA-RR loop has not yet been fully clarified. Therefore, our aim was to identify the neurochemical composition and connectivity of the VTA in comparison to that described for the rat brain.

2. In an attempt to identify further putative locations for the avian 'prefrontal cortex', a further pallial candidate, the *rostral Wulst,* was studied with respect to its subcortical connectivity and neurochemical character.

3. We sought to investigate the distribution, target neurons and transmitter colocalization of the putatively serotonergic mesopontine afferentation to the VTA.

4. We attempted to describe the synaptic morphology of 5HT immunoreactive afferents as well as shedding light on the yet unknown ultrastructure of the avian VTA.

III. Experimental procedures, subjects and methods

COMPARATIVE STUDY ON THE DISTRIBUTION OF SEROTONINE, TYROSINE HYDROXYLASE AND DARPP WITHIN THE OF MONOAMINERGIC NUCLEI OF RATS AND DOMESTIC CHICKENS

Young domestic chicks (*Gallus domesticus*) and young albino rats of both sexes were used.

Preembedding immunocytochemistry

Following terminal anaesthesia, the animals were transcardially perfused with 4% paraformaldehyde in 0.1M PB (pH 7.4), the brains then harvested and blocked.

Fifty to seventy micrometer thick free-floating Vibratome sections, containing the mesopontine or cortical regions, were selected. We employed single or combined immunocytochemical (tyrosine hydroxylase - TH, serotonin - 5-HT, DARPP-32) stainings to visualize the neuronal markers within the avian or mammalian VTA, RR, ACC and mPFC. Double immunolabelling was assessed using fluorescent secondary antibodies.

Reagent dilutions

TH - 1:6000 (mouse monoclonal AB, EugeneTech and Incstar), for immunofluorescence 1:1000;

5-HT - 1:60000 (rabbit polyclonal AB, Incstar), for immunofluorescence 1:5000;

DARPP – 1:30000 (mouse monoclonal AB, gift from H.C. Hemmings), for immunofluorescence 1:5000.

All antibody dilutions were made in 0.01 M PBS containing 1% NGS.

For DAB reactions 0.05 M TRIS-HCl solutions (pH 7.5 or pH 8.00) were used.

ANTEROGRADE AND RETROGRADE PATHWAY TRACING WITHIN THE WULST-VTA SYSTEM

One week old chicks of both sexes were used for the tracing experiments. The birds were anaesthesised with a mixture of Ketamin-Rompun and mounted on a modified stereotaxic frame.

Anterograde tracing

Twelve one-week-old chicks were subjected to anterograde tracing experiments, with six birds forming each experimental group.

Group (1) birds received iontophoretic injections of 5% Phaseolus vulgaris Leucoagglutinin (PHAL) in the rostral Wulst as described in Székely and Krebs (1996).

Group (2) chickens were pressure injected with 10% BDA in the ventral tegmental area as described in Mezey and Csillag (2001).

Following 21 days or 14 days of survival the PHAL or BDA injected chicks were terminally anaesthesised and perfused with a sodium phosphate buffer containing 2% paraformaldehyde and 1% glutaraldehyde (pH 7.4). Following postfixation, the brain blocks were sectioned at 70 micrometer on a vibrating microtome and the sectiones were processed according to that described in Székely and Krebs (1996) and Mezey and Csillag (2001). The immunocomplex was visualized by tyramine

amplified Ni- ammonium sulfate diamino benzidine reaction (Ni DAB). The sections were the mounted, dried and covered in DepeX to be viewed in an Olympus Vanox photomicroscope.

Retrograde tracing

Unconjugated Horseradish peroxydase (HRP) was used as a retrograde tracer. Five chickens were pressure injected in the rostral Wulst with 30% HRP using the same stereotaxic coordinates as for the anterograde tracing studies. Following two days of survival, the birds were terminally anaesthesized and perfused with a sodium buffer solution containing 1% paraformaldehyde and 3% glutaraldehyde (pH 7.4) Following postfixation, the brains were blocked and 70 micrometer sections were cut on a vibrating microtome which then were reacted in a solution containing nickel ammonium sulphate and diaminobenzidine to reveal the reaction sites. The sections were the mounted, dehydrated and covered in DePeX to be viewed and photographed in an Olympus Vanox photomicroscope.

Preembedding immunocytochemistry

Primary antisera against Substance P (SP), tyrosine hydroxilase (TH) and DARPP were used. Spare sections containing the VTA, Mst/ACC and Wulst of the aboves series were reacted for immunocytochemistry to visualize the neurochemical, perykarial or axonal, markers.

SP - SP 1:500 (rabbit polyclonal; Zymed)

TH - 1:6000 (mouse monoclonal AB, EugeneTech and Incstar);

DARPP – 1:30000 (mouse monoclonal AB, gift from H.C. Hemmings);

All antibody dilutions were made in 0.01 M PBS containing 1% NGS.

For DAB reactions 0.05 M TRIS-HCl solutions (pH 7.5 or pH 8.00) were used.

DESCRIPTION OF SEROTONERGIC, DOPAMINERGIC AND NITRERGIC NEURAL ELEMENTS IN THE BRAINSTEM NUCLEI OF THE DOMESTIC CHICKEN AND THE ZEBRA FINCH

Commercially available adult zebra finches (*Taenopygia guttata*) and young domestic chicks (*Gallus domesticus*) were used. Following terminal anaesthesia, the birds were transcardially perfused with 4% paraformaldehyde in 0.1M PB (pH 7.4), the brains then harvested and blocked

NADPH-diaphorase histochemistry

5HT and tyrosine hydroxilase immunocytochemistry

Seventy micrometer thick free-floating Vibratome sections, containing the mesopontine region, were selected. We employed combined immunocytochemical (tyrosine hydroxylase - TH, serotonin - 5HT) and histochemical (NADPH-diaphorase - Nd) techniques to verify the neuronal markers in the avian AVT and presumed dorsal raphe region.

Reagent dilutions

TH - 1:3000 (mouse monoclonal AB, EugeneTech and Incstar),

5-HT - 1:8000 (rabbit polyclonal AB, Incstar),

NADPH - 10% (Sigma), NBT- 1% (Sigma)

All antibody dilutions were made in 0.01 M PBS containing 1% NGS.

For enzyme histochemistry and DAB reactions 0.05 M TRIS-HCl solutions (pH 7.5 or pH 8.00) were used.

ELECTRONE MICROSCOPICAL OBSERVATIONS ON THE ULTRASTRUCTURE OF THE VTA

One week old chicks of both sexes were used for the experiments

Following terminal anaesthesia, the birds were transcardially perfused with a fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1M PB (pH 7.4). The brains were then harvested and blocked

Preembedding serotonin immunocytochemistry

Seventy micrometer thick free-floating Vibratome sections, containing the mesopontine region, were reacted with an anti-5-HT antiserum (1:20000 rabbit polyclonal AB in 0.01 M PBS, Incstar), then the immunoprecipitate was visualized with DAB.

Electron microscopy

Both 5-HT reacted and 'empty' sections were osmicated and embedded in Durcupan.

Ultrathin section were cut on a Leica Ultracut and viewed in a JEOL electrone microscope

III. Results and conclusions

1. Neurochemical characterization comparison with the rat brain

In general, VTA, RR, ACC and mPFC exhibit an almost identical neurochemical character in both vertebrate classes, however, some minor alterations are apparent. **The ventral tegmental area contains more subdivisions in the rat brain than the avian VTA, where we may only distinguish two, albeit not clearly separated, subregions (shell and core).**

The rat VTA is enmeshed by a profuse network of 5-HT fibres, but neuronal somata are never seen. Rather surprisingly, **the avian VTA appears to contain a specific fusiform neuronal type apparently exhibiting 5-HT immunoreactivity.**

The raphe nuclei contain more DARPP immunopositive fibres in the chick brain than in the rat. Similar to that found in the rat mPFC, both the PLT and the **rostral Wulst contain numerous DARPP neurones as well as a dense network of TH or 5-HT labelled fibres**.

Therefore, it is suggested that the avian prefrontal cortex may involve the rostral hyperpallial areas together with the PLT and the dorsolateral cortex.

2. Tract tracing and prefrontal cortical homology

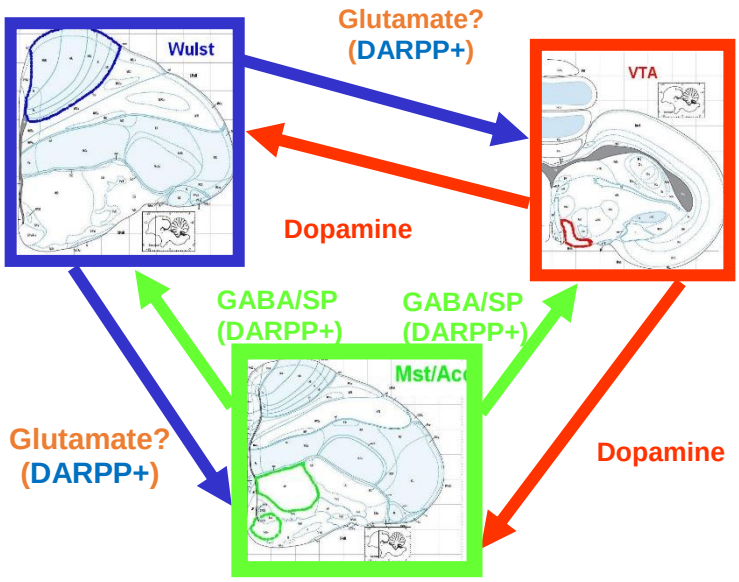
The rostral Wulst is part of the 'avian prefrontal complex'

Retrograde (HRP) and anterograde (BDA, PHAL) tracer injections into the anterior Wulst have proven the existence of a preferentially ipsilateral loop between the Wulst and the ventral tegmental area. There was a moderate contingent of anterogradely labelled varicose axons traversing the ipsilateral VTA, whereas retrograde tracer injection resulted in a bilateral appearence of backfilled perikarya.

Furthermore, the rostral Wulst may be homologous to part of the anterior cingulate, infralimbic and prelimbic cortices of the rat brain

The pattern of efferent projections of the rat mPFC, studied by retrograde and anterograde tracing techniques, suggests a widespread territory of VTA projecting neurones. A minor contingent of layer 5 output neurons (4-6 %) in IL, PL, and ACd projects to VTA, with the highest number occurring in PL. (Gabbott et al 2005)

The rostral Wulst, the Acc core and the MSt are interconnected with each other (see the diagram below) The ipsilateral nucleus accumbens (Acc) and the medial striatum (MSt) are also heavily enmeshed by anterogradely labelled varicose axons arising from the VTA, thus confirming the presence of a dopaminergic ventral tegmental afferentation, implicated in reward mechanisms.



Evidence indicates in the mammalian brain that dopamine transmission in the (Acc) is modulated by glutamatergic projections from the medial prefrontal cortex (mPFC) to Acc and VTA (Doherty and Gratton, 2007). It also seems, that corticofugal input to Acc indirectly regulates stress-induced dopamine release through a GABA feedback pathway to VTA.

According to our hypothesis, in birds, these cortical subregions comprise not only the posterolateral telencephalon (PLT) or the medial nidopallum (MNH) but also the rostral Wulst, being connected to the midbrain dopaminergic nuclei as well as having a role in stress response (Müller and Scheich, 1983).

Both the VTA, with mainly in its shell region, and the rostral Wulst contain a profuse substance P (SP) reactive axonal network, presumably deriving from MSt. Thus suggesting for SP an important role in stress-induced activation of mesocortical dopamine neurones.

Several lines of evidence implicate the neuropeptide SP in the modulation of emotional behaviour. Stress will stimulate dopamine transmission in both the mPFC and the Acc (Abercrombie et al.1989), e.g. in rats, footshock stress may activate mesocortical dopaminergic cells, while also decreasing SP concentrations in the midbrain interpeduncular nucleus and the adjacent VTA, but not in the substantia nigra (Bannon et al, 1983). This suggests that the activation of the SP input to the VTA may mediate activation of certain DA systems by footshock stress; behavioural studies also had suggested an excitatory effect of SP on DA cells in the VTA. Similarly, in drug seeking behaviour it has been suggested (McFarland et al. 2004) that footshock stress activates the limbic circuitry of the central extended amygdala, including the central nucleus, the ventral bed nucleus of the stria terminalis and the Acc shell, that, via the VTA, will activate the motor output circuitry.

3. Tyrosine hydroxylase (TH) immunopositive, nitrergic (Nd) and serotonergic (5HT) neural elements in the VTA.

Large Nd reactive cells are found preferentially to occupy the VTA shell of the domestic chicken and the zebra finch. Worth noting, that only a minor contingent of neurons colocalize Nd and TH within the VTA. Earlier studies have shown approximately a 40% presence colocalization TH and Nd within the mesencephalic and pontine neurons of the Japanese quail (Panzica et al 1996) similar to that found in the lizard brain by Smeets et al. (1997).

A profuse network of 5HT positive axons enmesh the AVT and perisomatic contacts are formed within the entire nucleus. The mammalian ventral tegmentum is reciprocally connected to the mesopontine raphe nuclei thus receiving a major inhibitory pathway (Dun et al. 1994) arising from both 5HT positive, or to a lesser extent, 5HT negative raphe neurons.

The putative avian raphe nuclei (nucleus linearis caudalis, LC) contain a high number of 5HT positive neurons and exhibit a scarce appearence of Nd reactive cells. **Similarly**, **only a small percentage of 5-HT positive LC neurons colocalize Nd.**

In the rat, the majority of Nd reactive neurons in the dorsal/median raphe nuclei are 5HT positive, whereas very few 5HT cells are Nd positive in the more caudal midline entities (Dun et al. 1994).

TH positive elements show a peculiar distribution in LC with the rostral aspect containing TH positive perikarya, whereas in the caudal LC, only varicose fibers are apparent.

It has been suggested by Stratford and Wirtshafter (1990) that the projection targets of TH positive cells of the dorsal raphe resemble those of the dopaminergic cells of the VTA, rather than those of the serotonergic cells within the DR.

Numerous LC 5HT positive axons surround unlabelled (presumably GABAergic inhibitory) somata in the VTA, whereas those boutons that are juxtaposed to chemically identified cells, preferentially target TH containing, rather than Nd reactive, cells of the AVT.

It has also been verified, that the inhibitory nature of 5HT projections may trigger an initial excitation in approximately two thirds of the dopaminergic population of AVT, presumably via disinhibition, and only the rest of AVT neurons respond with an inhibition (Gervais and Rouillard 2000).

4. Distribution and synaptic morphology of 5HT immunoreactive afferents; electrone microscopical structure of the avian VTA.

A profuse serotonergic network has been found in the entire extent of the AVT, forming baskets around both TH immunopositive and negative perikarya as shown by double immunofluorescence, as well as Nd reactive cell bodies and dendrites. Only a minor contingent of AVT neurones appeared to colocalize TH and Nd. Nevertheless, the existence of an external nitrergic influence cannot be excluded, since the majority of the 5-HT immunoreactive neurones also express Nd in the rat DRN. Although the presumed avian DRN homologue, nucleus linearis caudalis, contained fewer neurones colocalizing Nd and 5-HT, the existence of a similar inhibitory loop between the AVT and DRN is suggested.

Terminal varicosities containing round synaptic vesicles have contacted small and medium size dendritic profiles of, presumably, principal cells of VTA. The large boutons engulf the dendritic portions forming long appositions. Primarily, the synaptic vesicles appear to be small and clear, however, in some cases, dense core vesicles are also apparent. The boutons seem to predominantly form asymmetrical synapses, however, in some cases, long appositions lacking recognised densities are also observed.

The 5-HT immunoreactive synaptic terminals are stuffed with **clear and round vesicles**, however, they may also contain a few dense core vesicles.

A complex synaptic feature is observed frequently, where the central position of the dendrite, surrounded by terminals, suggests the presence of numerous glomerular synapses.

Boutons unlabelled for 5-HT and forming excitatory synaptic contacts with the principal cells of VTA may contain acetyl choline or glutamate (from mPFC) as described for the mammalian brain.

IV. Major findings

1. The avian ventral tegmental area contains only two, albeit not clearly separated, subregions (shell and core) while the mammalian homologue may be subdivided into several subregions. The shell contains predominantly large nitrergic neurons, while the core seems to be devoid of such cells.
Telencephalic SP or DARPP positive axons arborise profusely in the nucleus thus suggesting a reciprocal innervation among the centres.

2. We described a mainly ipsilateral neuronal loop between the VTA and the rostral Wulst, thus completing the collection of telencephalic pallial regions, thought to be equivalent to the rodent medial prefrontal cortex. Furthermore, due to its specific connectivity, the rostral Wulst may correspond to certain specific regions of the mammalian mPFC, including the anterior cingulate, infralimbic and prelimbic cortices.

3. We verified a reciprocal connection between the VTA and the mesopontine serotonergic nuclear complex (nucl. linearis caudalis) thought to be homologous to the dorsal/median raphe nuclei.

4. We described the distribution of serotonergic axons forming baskets around dopaminergic or unlabelled principal cells of VTA. Furthermore, we detected an unusual, in mammals yet unidentified, group of small, fusiform, serotonergic neurons within the caudolateral aspect of the nucleus.

5. We described the ultrastructure of the avian ventral tegmental area, and we have found 5HT labelled terminal varicosities containing round synaptic vesicles contacting small and medium size dendritic profiles of, presumably, principal cells of VTA. The boutons seemed to predominantly form asymmetrical synapses, however, in some cases, long appositions lacking recognised densities were also observed. Furthermore, we are the first to describe the presence of complex synaptic structures with several terminals forming contact with the same dendrite, thus suggesting the presence of glomerular synapses within the VTA.

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A serotoninerg bemenet tárgyalásából kiindulva mutatom be az **area ventralis tegmentalis (Tsai)** (vagy ventral tegmental area = VTA) kapcsolatrendszerét, neurokémiai és elektronmikroszkópos karakterizálását madárban, összehasonlítva az emlősben talált eredményekkel.

A VTA az A10 monoaminerg magcsoportnak felel meg, a n. oculomotorius kilépésénél találjuk a középagyi tegmentumban. Ez a mag részt vesz egyes tanulási folyamatokban valamint az addikciós viselkedések hedonisztikus komponense is ideköthető. A VTA így a jutalmazási kör része, többek között a hippocampus-szal, nucleus accumbens-szel, mediális striátummal, prefontális (mPFC) kéreggel áll kapcsolatban (ennek bizonyítása történt BDA, HRP, PHAL pályajelöléssel). A reciprok kapcsolat feltárásával azt is bizonyítjuk, hogy a homológia kérdését nem figyelmen kívül hagyva, viszont funkcionális szeparációt feltételezve, a **madáragy palliumának több területéből** áll össze az **emlősben egységes** területként található mPFC.

A mag neurokémiai vizsgálatának során megállapíthatjuk, hogy az accumbens mellett a VTA (főleg a shell regio) gazdag Sustance P+ arborizációt tartalmaz, ugyanitt palliális eredetű DARPP-jelölt rostokkal is találkozunk. **Az agytörzsi raphe-magokból serotoninerg afferentáció éri el a VTA-t.** 5HT immuncitokémiával a VTA két alegységében érdekes jelölés-eloszlást látunk. Az elülső területen csak rostok jelennek meg, de hátrébb, a **caudolateralis részében kis fusiformis 5HT-sejtek tűnnek fel** (ilyen nincs az emlősben). Az elülső, rostromedialis részen viszont csak 5HT rostok vannak, ezek a TH - (tirozin hidroxiláz= dopaminerg) -pozitív (és negatív) valamint kisebb mértékben a NADPH-diaforázzal (Nd) jelölt neuronok körül kosárszinapszisokat hoznak létre.

Ugyan a VTA-neuronokban a TH és Nd kolokalizáció kismértékű, viszont a nitrerg rostok hatása emlősben igen fontos (a VTA-ba projiciáló raphe dorsalis 5HT- neuronok részben nitrergek), sajnos madárban csak kisfokú kolokalizációt figyelhetünk meg a középagyi raphe magcsoportnak megfelelő nucleus linearis caudalis TH-sejtjeiben.

A VTA bemenetének egyik legfontosabb komponense a raphe-komplexumból származó 5HT+ (főleg gátló hatású) pálya. A dorsalis raphe 5HT sejtjei GABA-t kolokalizálhatnak.

Konfokális mikroszkópos megfigyelések szerint **a VTA területén található 5HT+ rostok a TH-sejtek szómájának és dendritjeinek a közelében számos közeli kapcsolatot (close contact) hoznak létre** ezzel szinapszis jelenlétét feltételezve.

Az 5HT+ terminálisok EM-mel vizsgálva **kis kerek, tiszta vesiculákat** tartalmaznak (egy-egy dense core vesicula elvétve előfordul), és általában a principális sejtek dendritjeivel alkotnak szinapszist. **Főleg aszimmetrikus szinaptikus profilokat látunk, de előfordulnak a dendriteket átölelő/kísérő hosszú appozíciók, kifejezett szinaptikus denzitás nélkül is.**

Jellegzetesen sok dense core vesiculát tartalmazó terminálist látunk. Emellett találunk jelöletlen terminálisokat is, amelyek a (feltehetően) principális sejtek dendritjeivel aszimmetrikus szinaptikus profilokat hoznak létre. Feltételezéseink szerint ezek glutamaterg végződések, amelyek az mPFC-nek megfelelő területről (*anterior Wulst vagy PLT*) származnak.

Jellegzetes komplex szinapszisokat (glomerularis szerkezetet) is látunk, egy-egy dendrit körül több szinaptikus terminális helyezkedik el, aszimmetrikus szinapszist alkotva (mind kis, kerek vezikulákkal, mind pedig 'dense core' vesiculákkal).

A tanulási folyamat egyik fontos eleme a pozitív megerősítés és a VTA, mint a jutalmazási kör fontos eleme a kortikális területekkel mind közvetlen, mind pedig a szubkortikális területeken (Acc, MSt) kapcsolatban áll. A VTA-ból származó dopaminerg efferentáció nucleus accumbensre kifejtett hatását mind a prefrontális kéregből, mind pedig a mesopontinus raphe-komplexből érkező visszacsatolás modulálja, ennek elemei a kérgi kolinerg /glutamaterg serkentő axonok, és az agytörzsi, elsődlegesen gátló bemenet. Mindemellett megállapíthatjuk, hogy a sok hasonlóság ellenére a gerinces osztályok között (de az emlősök között is) jelentős eltérések tapasztalhatók az azonos agyi területeket összekötő hálózatok neurokémiájában, ami azt bizonyítja, hogy az evolúció során a központi idegrendszer egy adott feladat sikeres elvégzéséhez több alternatív, de egyenértékű mechanizmust is felkínál.