Expression of the tyrosine hydroxylase gene in nucleus accumbens (NAc) and ventral tegmental area (VTA) in the mouse brain

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Abstract

Brain represents a ‘master mind’ of the rest of the body, and during the evolution, different surviving mechanisms emerged to help organisms to fit best into their environment. Reward pathway of the brain is one of many specifically arranged networks in the brain that is responsible for orchestrating animal behavior. Neurotransmitter dopamine is released and signal cascade is established in between key players of the pathway – ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC). Tyrosine hydroxylase (Th) is an enzyme that represents a limiting factor and is responsible for dopamine synthesis. Since dopamine is only synthesized in the VTA, tyrosine hydroxylase mRNA is not expected to be found anywhere else in this pathway, rather than in the VTA. If found, it is expected to be present in low levels, and that is already known from the literature. The goal of this study was to confirm that I isolated correct regions of the brain and I was checking if Th is upregulated in VTA versus NAc, as expected from the literature. RNA from wild type male mice VTA and NAc was subjected to RT-PCR analysis, using different primer pairs. Both primer pairs in all of the subject animals gave similar results, corroborating literature data that there is indeed higher level of tyrosine hydroxylase expression in VTA than NAc.
Introduction

Most active and motile animals show some kind of foraging behavior and are able to respond to a stimulus. A stimulus may be harmless and animals may respond with approaching behavior to seek for some kind of benefit. In the same manner, a stimulus may be aversive and the animal may try to escape to avoid it. These are some of the basic behavioral responses that developed during the evolution, and the field of behavioral research uses it to further explore neural circuits that are responsible for such evolutionary setups and adaptations. Pioneering researchers in behavioral science defined rewarding and punishing stimuli simply by the nature of the response it elicits – reward elicits approaching, while punishment elicits avoidance (Skinner, 1938). Reward learning and reward seeking behaviors are actions that represent fundamental aspects of animal behavior, and are universal across animals (Barron et al., 2010).

Neurobiological research of the reward responses and reward processing in mammals established structures and regions of the brain that are a part of reward pathway and these align nicely with the function they have. These structures are a part of mesolimbic dopaminergic pathway, which is located ‘deep in the brain’ indicating how early in the evolution these structures developed and these properties emerged, also indicating their important role for survival. Main structures are ventral tegmental area (VTA) that projects its axons to nucleus accumbens (NAc), and further make connections with neurons of prefrontal cortex (PFC), which represents the conscious part of this pathway, and it evolved later in the evolution (Adinoff, 2004) (Figure 1A).

Neurotransmitter dopamine has been characterized as the key modulator of this circuit and also of the behavioral responses to rewards (Berridge and Robinson, 1998).

Tyrosine hydroxylase is the rate-limiting enzyme of catecholamine biosynthesis, in which group dopamine belongs, along with adrenaline for example. It represents a key step in dopamine synthesis and it is used to convert L-tyrosine into L-DOPA by tetrahydrobiopterin and molecular oxygen. Modes of regulating this enzyme’s activity include phosphorylation at 4 different serine residues by multiple kinases, and dephosphorylation by 2 phosphatases; the enzyme activity is also inhibited in feedback
fashion, by the catecholamine neurotransmitters (e.g. adrenaline, noradrenaline and dopamine) (Daubner et al, 2011). Since dopamine synthesis requires presence of the enzyme tyrosine hydroxylase, which is present in dopaminergic neurons, dopamine is synthesized in VTA and then signaling cascade is established through efferent VTA axon projections towards NAc and further to PFC.

**Figure 1** – **A** mouse brain reward pathway with main subregions labeled – ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC); **B** enlarged synapse in between presynaptic VTA neuron and posynaptic NAc neuron depicting dopamine receptor 1 and 2 (DRD1 and DRD2), dopamine transporter and tyrosine hydroxylase localization.

Different receptors and signaling molecules are involved in dopaminergic mesolimbic pathway besides tyrosine hydroxylase. Different dopamine receptors are present on the presynaptic (dopamine receptor 2 – regulates dopamine re-uptake; dopamine transporter – main receptor that regulates dopamine re-uptake from the synaptic cleft) and postsynaptic (dopamine receptor 1 and 2) neuron (Figure 1B).

Reward is described as a feeling that follows discrete stimuli and is providing enjoyment. Addictions are described as persistent, uncontrollable and destructive behaviors. Even though reward and addition were thought to share common neurobiological basis and both of these processes share overlapping neuroanatomical structures, they are characterized as distinct neurochemical processes.
Olds and Milner 1954. performed an experiment where rodents had the opportunity to self-administer electrical stimulation in various brain regions. They found out that specific brain regions were more stimulated as opposed to the others, and this experiment represented the base of modern understanding of brain reward mechanism, because it set a first stone in revealing which brain regions were involved in this basic behavior. Subsequent experiments over various decades revealed that the pathway involved was a mesolimbic one, which originates in VTA where dopaminergic cell bodies are situated, following their axons to NAc as the main efferent projection of VTA, but also including other parts of the limbic system (amygdala, stria terminalis and lateral hypothalamus).

Natural rewards as food or sex increase the extracellular concentration of mesolimbic dopamine. Different substances as alcohol, caffeine, nicotine, cocaine and other drugs of abuse often abused by humans lead to the same effect. Cocaine for example increases synaptic dopamine concentrations by blocking the presynaptic dopamine transporter receptors and dopamine re-uptake (Adinoff, 2004).

Since tyrosine hydroxylase is a molecular marker for dopaminergic neurons, and is only found in VTA in higher levels, and since a new protocol for these brain regions isolations was being developed in the lab, a molecular confirmation of the accurate brain region isolation was needed.

**Material and Methods**

**Animals**

3 month-old wild-type C57BL/6J male mice were sacrificed by cervical dislocation and brains were immediately taken and frozen in hexane to preserve the shape and structure organization, after which they were kept on the dry ice and stored at -80°C until following dissections took place.
**Brain region isolation**

Brains were placed on the dry ice and taken to cryostat were temperature was at -20°C constantly, so that the brains did not thaw too quickly, and the dissections were performed. Nucleus accumbens and ventral tegmental area were dissected from each brain using acrylic brain matrix for coronal sections that gave slices of 1mm in depth. With guidance of the Mouse Brain Map Atlas, cuts were made and nucleus accumbens was punched first with 1mm punchers, afterwards slices for cutting out ventral tegmental area were made and it was punched with 1.5 mm diameter puncher. For each animal, each structure was dissected bilaterally and pulled. Immediately after that step, structures were snap frozen in liquid nitrogen until further RNA extraction steps.

**RNA Extraction and purification**

Both tissues were homogenized using light weight tissue homogenizer (Fisherbrand 150 Handheld Homogenizer Motor and Soft Tissue Omni Tip Plastic Homogenizing Probes) with addition of lysis buffer RLT lysis buffer containing β-mercaptoethanol. Then, from all of the lysates DNA and RNA were extracted using All Prep Mini Kit from Qiagen. Further, only the RNA was used for the subsequent analyses. The RNA levels of purity and concentration were checked using UV spectrometry and 5 ng/μg dilutions stocks were made and stored at -20°C for further use.

**RT-PCR (reverse transcription polymerase chain reaction)**

Reverse transcription and PCR reaction were performed in one-step using Qiagen One-Step RT-PCR Kit. 10μl volume reactions were made with 10ng of RNA in each reaction and primer concentrations were always 0.5 uM.

Primers were specifically designed in this project for tyrosine hydroxylase (Th):

For amplifying the region from Exon3-Exon11:

exon3F: GCAGTGCCAGAGGGCAAG

exon11R: TGCACCGTAAGCCCTCAGC

giving the product of **704bp** in length.
For amplifying the region from **Exon3-Exon4**:

- **Exon3F**: TACTTTGTGCGCTTCGAGGTG
- **Exon4R**: CAGGTGGTGACACTTATCCAAC
giving the product of **150bp** in length (Figure 2).

For amplifying the region from **Exon7-Exon8**:

- **Exon7F**: GCTTCTGGAACGGGTACTGTG
- **Exon8R**: AGAAAATCACGGGCAGACAG
giving the product of **132bp** in length (Figure 2).

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**Figure 2** – Schematic representation of tyrosine hydroxylase gene with exons and introns depicted; two primer pair design locations and expected product lengths are also depicted; source: [http://wormweb.org](http://wormweb.org)

RT-PCR for the housekeeping gene **Cyclophilin A (CypA)** was performed with 0.5uM of the following primers:

- **CypAF**: GAGCTGTTTGCAGACAAAGTTC
- **CypAR**: CCCTGGCACATGAATCCTGG
giving the product of **125bp** in length.

Visualizations of the products were done in a 1% agarose gel with Ethidium bromide.

Results were quantified using densitometric quantification method via ImageJ.
Products were then gel purified using the QIAquick Gel Extraction Kit or PCR product purified using QIAquick PCR Purification Kit and sent out for Sanger sequencing by Genewiz.

Sequencing results were further analyzed using NCBI BLAST.

Results
RT-PCR was performed with RNA from NAc and VTA brain regions (Figure 3A). Based off of the RT-PCR results, it appears there is more Th expression in the VTA as compared to NAc in the same animal. This was later confirmed using second pair of primers designed to a different region of the Th transcript. To ensure that this difference was not due to differences in the amount of total RNA in the RT-PCR reaction, I amplified CypA (cyclophilin A – a housekeeping gene), which showed no difference in expression between the two regions.

To quantify this difference I averaged my duplicates and took the ratio of the Th to CypA in NAc and compared that to the average ratio of the Th to CypA in the VTA. This analysis was done for both primer pairs using samples isolated from two different animals (Figure 3B).
Discussion

The main goal of this study was to determine levels of tyrosine hydroxylase expression in two brain regions – ventral tegmental area and nucleus accumbens, and in that way confirm accurate brain region isolation. The main interest in those structures comes from the fact that they are the key subcortical brain structures involved in formation of mesolimbic dopaminergic brain circuit, which is responsible for reward-seeking behavior and formation of ‘reward pathway’ (Adinoff, 2004). Since dopaminergic cell bodies, where dopamine is synthesized, are only situated in ventral tegmental area, expectations were that high level of expression will be observed in that region, as opposed to nucleus accumbens, which neurons only receive already synthesized dopamine and do not synthesize it themselves, and very low levels or tyrosine hydroxylase expression were expected in this region.

In Figure 3A, left and right panel, results acquired via RT-PCR that were later quantified are depicted, and those clearly showed corroboration of literature data and the fact that indeed in ventral tegmental area there is far more, around 2 folds (depending on the individual animal observed) more tyrosine hydroxylase mRNA expressed in comparison to nucleus accumbens. Still, more sensitive analysis, like quantitative RT-PCR should be performed to gain a more accurate understanding of the quantitative differences of this gene in the two brain regions.

If we look at the individual graphs in Figure 3B, showing data for individual animals, as well as results acquired with different primer pairs, there is an overall trend showing that in all animals with both primer pairs there is more tyrosine hydroxylase in ventral tegmental area than in the nucleus accumbens.

This data is in agreement with the literature, which reports that there is higher expression of tyrosine hydroxylase in ventral tegmental area as compared to expression in the nucleus accumbens.

These results also confirmed that the brain regions I harvested for the study were indeed ventral tegmental area and nucleus accumbens, since tyrosine hydroxylase is used as a
marker for determination of dopaminergic neuron bodies, only present in ventral tegmental area.

At the level of mRNA, high levels of tyrosine hydroxylase should only be detectable in ventral tegmental area, and only at the level of protein, tyrosine hydroxylase may be possible to be detectable in higher levels in both of these regions. There, for sure could be detectable a basic level of expression (constitutive expression of this gene is probably present) of tyrosine hydroxylase in nucleus accumbens, which explains that in the acquired results in this project, there is some level of tyrosine hydroxylase mRNA detected in nucleus accumbens as well.

Future studies on the protein level have to be conducted in order to acquire more in depth information and have a better picture about what is happening in this pathway beyond mRNA level.

References


