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Review

The Anxiolytic Effect of Intranasal Drug Administration in Exosomal Form of Oxytocin: Pro-Leu-Gly Amino Acids in Autism Spectrum Disorder

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ABSTRACT

Neurological disorders are complex and multifaceted health problems that can affect human life at any age, from infancy to old age. Their cellular mechanisms are complex and intricate. These disorders affect the brain, spinal cord, and nervous system and can significantly alter individuals' social behavior, cognitive abilities, and motor functions. Therefore, individual neurological disorders can have far-reaching effects at the societal level. Autism spectrum disorder (ASD) is a neurodevelopmental disorder that has been characterized by significant challenges in social interactions and repetitive behaviors, it affects approximately one in 100 individuals. The lack of effective treatment creates a substantial gap and leads to new research. Oxytocin (OXT), the neurohormone that is known for its regulative effects on social behavior, can be considered as a potential treatment for decreasing the anxiolytic effects of ASD. This review focuses on the use of OXT's neuropeptides Proline (Pro), Leucine (Leu), and Glycine (Gly) for minimizing the anxiolytic effects of ASD when applied as exosomes in intranasal form.

Keywords: Anxiety, autism spectrum disorder, exosomes, intranasal administration, oxytocin.

Neurodevelopmental disorders are neurological issues that can affect the central nervous system (CNS) of the patient. These disorders can create a negative impact on motor functions, cognitive behaviors, and emotional abilities. Neurodegenerative diseases are usually a result of the damage of nerve cells and they can develop due to inflammatory, genetic, environmental, or traumatic factors. Autism spectrum disorder (ASD) is a common neurological disorder that affects 1 in 100 people in the population.[1] It is recognized by the individual's lack of social communication, behavioral issues, repetitive behavior, and restricted interests.[2] It often comes with other neurological conditions like anxiety or intellectual disabilities.[3]

There is no known cure available for ASD, however, there are some treatment options discussed for reducing the symptoms of the disorder.[4] Oxytocin (OXT) is a neuropeptide that is known for its role in social behaviors, cognition, lactation, and maternal behavior. A recent study has shown that the OXT that is expressed by the paraventricular nucleus of the hypothalamus, reduction is correlated with aggression for male mice.[5] The observed effects of OXT have led the researchers to focus on exploring the potential use of this neurohormone to decrease the behavioral issues caused by ASD.[6]

In the treatment of neurological disorders, delivering macromolecular therapeutic agents across the blood-brain barrier (BBB) to the CNS poses a remarkable challenge. The extracellular vesicles (EVs) like exosomes have the ability to cross the BBB.[7] Exosomes are small EVs' surrounded by a bilipid layer.[8] When applied in intranasal form, which increases the efficient consumption and absorption of the desired drug, the use of the exosomal form of OXT's certain amino acids can be promising for the reduction of anxiolytic effects of ASD.

INTRANASAL DRUG ADMINISTRATION

Most large molecules with a molecular weight of more than 400 Da, more than nine hydrogen bonds, and insufficient lipophilicity, cannot cross the

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BBB. Therefore, drug development for neurological diseases is extremely hard because of the BBB which is an obstacle. When drugs are taken orally, the drug loses a significant amount of its efficacy while passing through the gastrointestinal system and also has difficulty in passing through the BBB. In contrast, intranasal drug administration allows high drug efficacy by bypassing the BBB with drugs that have a higher molecular weight (lower than 1000 Da) and have a lipophilic character. This method offers a non-invasive, relatively simpler route of drug administration and provides a faster effect compared to other routes. Additionally, it allows individuals with gastrointestinal issues to receive maximum benefit from the medication.[9] Thus, this method can offer efficient drug consumption for the old-aged individuals.

The nasal cavity is an advantageous site for the rapid and effective absorption of drugs into the body. One of the advantages is the presence of numerous blood vessels, which facilitates the passage of drugs into the bloodstream.[10] Moreover, the mucosa of the nasal cavity is permeable which aids drug absorption. Another important feature is that the olfactory nerves in the nasal cavity are directly connected to the brain.[11]

Drugs can reach from the olfactory sensory neurons to neuronal cells by entering the brain with the help of intraneuronal transport or by going into the cerebrospinal fluid (CSF) via perineuronal transport.[12] Thus, drugs can reach the brain directly through these olfactory nerves. Especially the olfactory bulb is at the center of these connections.[11]

According to Chung et al.[12], some early mouse studies show that the intracellular translocation times along the olfactory nerves of 1.5-6 hours or longer for up to 24 hours. Additionally, the respiratory region of the nasal cavity has trigeminal nerves, which provide another way for drugs administered through the nasal pathway to reach the brain.[11] Although the exact mechanism of how drugs are transported from the nasal cavity to the brain is not fully understood, the olfactory bulb and other carrier molecules in the nasal cavity can play a significant role in this process. This could help some drugs to directly reach the brain and cross the BBB.

Despite the positive aspects, there are some restrictions in the intranasal drug production and administration. Initially, the nasal cavity undergoes rapid mucosal clearance with the help of mucus which contains inorganic salts, antimicrobial enzymes, immunoglobulins, and glycoproteins. Antimicrobial enzymes and immunoglobulins of the mucus regulate immune responses against allergens and infectious particles, while glycoproteins support the viscosity and structure.[13] If the rapid mucosal clearance in the nose cleanses the nasal cavity before the drug is absorbed, the required dose of the drug may not be achieved. Moreover, the osmolality of nasal solutions should be between 290-500 mOsm/kg higher values may be tolerated in emergencies or for single applications. In addition, it should be avoided from the hypotonic formulations while isotonic or slightly hypertonic solutions are recommended.[14] Also, according to a study, the hypotonic form of nasal spray solutions can improve the permeability.[15]

The pH of the drugs is also an important factor for intranasal drug production. The nasal area has a slightly acidic pH (pH 5.5-6.5 in adults and 5-7 in infants).[10] Therefore, drugs should be developed by considering whether or not the drug is effective at a certain pH. Also, drugs should not be ionized and should not irritate the nasal mucosa.[14] If the body increases cleaning in the mucosal surface rapidly to dilute the irritant, it can prevent the drug from reaching the brain at an effective dosage. Another essential point is the bioavailability. The stability of intranasally administered drugs can be affected by the enzymes in the nasal mucosa. Drugs containing protein and peptides can be degraded by the proteases which are a part of an enzyme in the nasal mucosa or these peptides and proteins can increase molecular weight and reduce their permeability by binding immunoglobulins in the mucosa.[16] To preserve the drugs from enzymatic degradation, enzyme inhibitors may be used. The necessary volume of drugs that can be taken intranasally for an individual for once is an issue that needs to be considered. The optimum volume is 100 µL per nostril for droplets spread out the nasal cavity with the help of surface tension of the droplets and mucus layer.[15] If the volume exceeds the capacity of the nose, the applied preparations may be partially swallowed or simply flow out of the nose. This can prevent the intake of the desired dosage of this drug.

Oxytocin

Oxytocin is a neurohormone that is connected with social cognition and behaviors. It is produced in the supraoptic nuclei of the hypothalamus, and released to the bloodstream by the posterior pituitary gland. The influence of CNS happens through dendritic and somatic release of the OXT neurons. The release is dependent on the stimuli, like reproductive or

stress-related factors. In humans, there is a single type of oxytocin receptor (OXTR), whose expression in neurons and glial cells is regulated through various mechanisms, involving epigenetic modifications, availability of ligands, hormonal changes, and stress exposure.[17] According to Lim et al.[18], rodents who lack the OXT gene have been found with social deficiency. Another study showed that the escalation in the OXT levels has a positive impact on social bondings.[19]

Oxytocin is characterized by its unique structure. The first six amino acids of the OXT come together to form a ring shape because the two cysteine amino acids bring together with a disulfide bond except for these last three amino acids of the OXT which are pro-leu-gly (PLG) and connected to the ring shape as a short tail.[20,21]

While the cyclic section of the OXT molecule penetrates deeply into the transmembrane core of the OXTR, the short tail of the OXT molecule interacts with regions of the receptor located in the extracellular space.[21]

Moreover, PLG has several promising behavioral aspects such as inducing some maternal behavior. Most importantly, it interacts with opioid receptors resulting in stronger dopaminergic functions. Studies have shown that low dosages of PLG injection inhibit spontaneous motor activity and lower rats' diastolic blood pressure. Furthermore, high dosages of PLG markedly lowered gastrin and OXT levels in the plasma.[22] When the results of this experiment are considered, it can be seen that PLG has remarkable impacts on the neurological system by affecting blood pressure, hormones, and spontaneous motor activity. Reduced hormone levels and blood pressure point to a potentially soothing effect from an anxiolytic standpoint, while decreased spontaneous motor activity points to a potentially calming and anti-stress effect. For example, Moberg et al.[23] performed two experiments with OXT fragments: one in which the cyclic portion of OXT was preserved while the tripeptide extension PLG was modified, and another in which the cyclic structure was linearised but the carboxy-terminal end PLG was retained. In the first case, there was no significant difference in spontaneous motor activity when compared to the control group. However, when the cyclic structure was opened and the carboxy-terminal end was retained, there was a decrease in motor activity when compared to the control group. This finding is compatible with a sedative or anti-stress effect.

The nine amino acid neuropeptide OXT(Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly), modulates various physiological functions and behaviors.[24] These behaviors include aggression, response to social stimuli, recognizing emotions, maternal behavior, sexual acts, and making decisions. Overall concentrations of amino acids' biological availability may affect the pathogenesis of psychiatric disorders.[25]

The three amino acids, Pro, Leu, and Gly of OXT are key for the functioning of the oxytocinergic system.

Pro plays a significant role in the structural dynamic of the OXT molecule. The change in the position of this amino acid causes a crucial structural alteration for OXT.[26] Pro is responsible for the stabilization and folds of the protein.[27] Placement of the Pro in the 8th amino acid position causes the alteration of the OXT molecule. When altered, receptor interaction and activation become specific. A study from 2014 has found that this structural change causes the OXT signaling pathways that control the social bonding, mating, and overall sociosexual behaviors in marmosets.[28] This shows that the behavioral influence of the Pro results in diversity of the oxytocinergic system.

Leu, one of the branched-chain amino acids plays a crucial role in the brain by entering the brain through the capillary/glial interface.[29] It involves several neurological mechanisms that depend on its concentration.[30] Initially, Leu functions as an amino group donor by undergoing transamination to form glutamate in astrocytes.[29] Leu, with an α-ketoisocaproate carbon skeleton, is released into the extracellular fluid and taken up by neurons after amino group donation.[29] Aside from its effect on the glutamate-glutamine cycle, Leu regulates enzymes involved in energy metabolism in the brain and works as a signaling molecule in providing fuel material to energize the entire organism.[30]

Gly is a neuropeptide that is historically known as a minor neurotransmitter in the forebrain. It has an important role in the CNS when regulating social behaviors. Gly receptors are correlated with the development of the brain.[31,32] Research from Ito et al.[33] indicated that during the early postnatal period, Gly acts similarly to gamma-aminobutyric acid (GABA), initially showing a stimulating effect and then playing an inhibitory role as the brain develops. This transition is vital for correct neural development and the formation of functional neural circuits linked to social interactions. Additionally, just like GABA, Gly

may function as a stimulant neurotransmitter, it can activate chloride channels during early development, causing depolarization of membrane potentials due to high intercellular chloride levels. Moreover, a study by Qu et al.[34] suggests that Gly's protective effects against oxidative stress play a role in maintaining CNS health, by preserving the integrity of neural pathways involved in social cognition.

Overall, the disturbance of the oxytocinergic system may play an important part when it comes to neurological disorders with behavioral incapacitation.[35] Therefore, the oxytocinergic system is a significant research subject when it comes to a potential therapeutic against the anxiolytic effects of ASD.

Exosomes

Exosomes are small EVs surrounded by a bilipid layer that can be found in tissues and biological fluids such as plasma, breast milk, amniotic fluid, saliva, and CSF.[36,37] Due to their release from various cell types and their presence in biological fluids, EVs are involved in many cellular functions. Clearing cellular waste and facilitating the transfer of exogenous substances such as proteins, mRNA, miRNA, and lipids are some of their duties.[38,39]

Exosomes maintain intercellular homeostasis and play a role in intercellular communication.[40] They also carry some substances of the donor cell. Proteins, lipids, and mutations of the donor cell can also be seen in the exosome.[41] According to a study from Zhang et al.[42] exosomes, which have a cargo of cell-specific proteins, lipids, and nucleic acids, may serve as an intercellular communication mechanism. Extracellular vesicles can communicate by functioning both locally and at a distance. Once released, EVs interact with their environment, delivering substances to specific cells and altering their properties. Moreover, EVs have the ability to cross difficult biological barriers, such as the BBB.[43] This property of the exosomes can become beneficial to the detection and treatment of neurological diseases.

Exosome formation begins with the production of endocytic vesicles from the cell membrane through endocytosis, forming an early endosome (EE). As the EE matures into a late endosome (LE), its limiting membrane invaginates and forms vesicles within the lumen. The accumulation of these intraluminal vesicles within LE's is referred to as multivesicular bodies.[44] The LE can follow two different pathways: degradation and secretion. Part of the LE is directed toward lysosomes where its contents are degraded and the other part moves toward the plasma membrane and fuses with it, releasing the contained exosomes outside the cell. Then, exosomes released outside the cell can be taken up by recipient cells.[45] They can fuse directly with the recipient cell membrane or the recipient cell can engulf the exosome through endocytosis.[44]

To create a drug using the mechanism of exosomes and use them as desired, they must first be produced in the laboratory artificially and modified. Artificially produced exosomes can be generated in several ways. Mainly used artificial exosomes are called nanovesicles (NVs) which are produced using a top-down strategy, similar to natural exosomes.[46]

Initially, cells are disassembled, resulting in the creation of new NVs with a membrane structure similar to that of natural exosomes. These NVs carry proteins, nucleic acids, or lipids found in natural exosomes, thereby exhibiting characteristics similar to biological diversity.[47] While they replicate the complexity of natural exosomes, their production requires more time and effort. Exosome-mimics are another type of artificial exosome produced using a bottom-up strategy.[46] In this strategy, small molecules are used as building blocks and then these complex structures are created by combining their physical and chemical properties.[47] They are suitable for large-scale production and have lower production costs, but they do not replicate the biological complexity of natural exosomes as well. The other type is the hybrid exosomes which are produced with a bio-hybrid strategy and contain natural exosome components.[46]

Although natural vesicles are used in this method, synthetic nanoparticles are also involved.[47] They offer higher delivery efficiency than liposomes and greater stability than exosomes, but their production yield is lower.[46]

Intranasal Application of Exosomes

In the nervous system, exosomes can attend the intercellular communication, protect myelin, and remove wastes. They are involved in abnormal pathological processes like CNS diseases. Likely, they also play a role in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, traumatic encephalopathies, and prion disease.[48]

To clarify the therapeutic potential of exosomes in neurological diseases, it is necessary to determine how they cross the BBB and to develop methods for their isolation, loading, characterization, and

targeting of different cell types. The greatest challenge in this application is crossing the BBB, which can potentially be achieved through intranasal administration. It is a subject that is studied due to the advantages of the use of intranasal exosomes. There are many experiments carried out using this intranasal exosome mechanism, especially experiments about brain-related diseases. For example, when curcumin, which is a powerful anti-inflammatory agent, was prepared exosomal (exo-cur) and given to the mice whose brain's inflammatory response was triggered by LPS. When the mice that took exo-cur every day for 31 days observed, it was seen that the number of activated inflammatory microglial cells decreased. So inflammation decreased when exosomal curcumin was applied intranasally. Another example of exosomal drugs is CoQ10-loaded exosomes gained by adipose-derived stem cell usage in Alzheimer's disease in rats. As a result, these exosomes result in the improvement of cognitive and memory deficits by increasing the brain-derived neurotrophic factor and SOX2 in the hippocampus.[49]

Similarly, according to Huang et al.,[50] mesenchymal stem cell-derived exosomes (MSC-exos) help to recover neuroinflammation in Alzheimer's disease, which has neuroinflammation results from the accumulation of β-amyloid and also recovers spatial learning ability and memory impairment of AD transgenic mice. Moreover, they have anti-inflammatory effects by inhibiting the release of active microglia, reactive astrocytes, and cytokines. In another research, MSC-exos has been applied to mice that have disabled the Shank3B gene and therefore have ASD. When MSC-exo was applied to these mice intranasally, it was observed that mice spent more time in the chamber with the unfamiliar mice, whereas those treated with phosphate-buffered saline exhibited no particular preference in the three-chamber test. There were also similar results in the social novelty test.[51]

In conclusion, OXT and its Pro, Leu, and Gly neuropeptides have an important therapeutic impact on the socio-behavioral anxiolytic side of neurodevelopmental disorders like ASD. The intranasal application of this treatment is a non-invasive way to cross the BBB, however, this method has its limitations in drug emission and molecular size. Additionally, exosomal transportation presents a new strategy for delivering OXT and its related neuropeptides Pro, Leu, and Gly directly to the brain, although its clinical viability and safety require further research. These approaches have produced mixed results in current studies, this underscores the need for continued studies. The findings represent a significant step towards the use of OXT in the treatment of neurological disorders in the future.

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